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(71) Applicant *(for all designated States except US)*: MAX-PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Berlin (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: EATON, Suzanne [US/DE]; Reger Str. 2, 01309 Dresden (DE). THIELE, Christoph [DE/DE]; Birkenstr. 32, 01474 Dresden (DE). PANAKOVA, Daniela [SK/DE]; Louisenstr. 12, 01099 Dresden (DE). MAROIS, Eric [FR/DE]; Neubertstr. 27, 01307 Dresden (DE). SPRONG, Hein [NL/NL]; Gerrit Jan van der Veenlaan 11, NL-3705 PE Zeist (NL).

(74) Agent: VOSSIUS & Partner; Siebertstrasse 4, 81675 Munich (DE).

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(54) Title: TREATMENT OF HEDGEHOG- AND WNT-SECRETING TUMORS WITH INHIBITORS OF LIPOPROTEIN PARTICLE BIOGENESIS

(57) Abstract: This invention relates to the use of an inhibitor of Microsomal Triglyceride Transfer Protein (MTP), HMG-CoA reductase, DGAT and/or ACAT for the preparation of a pharmaceutical composition for the treatment of tumors. In a preferred embodiment, growth and/or progression of the tumor are caused by one or more protein of the Wnt or Hedgehog family. Preferred tumors are esophageal tumor, biliary tract tumor, gastric tumor, pancreatic tumor, malignant melanoma, colorectal tumor, squamous cell carcinoma and cervical tumor.

Treatment of Hedgehog- and Wnt-secreting tumors with inhibitors of Lipoprotein particle biogenesis

This invention relates to the use of an inhibitor of Microsomal Triglyceride Transfer Protein (MTP), HMG-CoA reductase, DGAT and/or ACAT for the preparation of a pharmaceutical composition for the treatment of tumors. In a preferred embodiment, growth and/or progression of the tumor are caused by one or more protein of the Wnt or Hedgehog family. Preferred tumors are esophageal tumor, biliary tract tumor, gastric tumor, pancreatic tumor, malignant melanoma, colorectal tumor, squamous cell carcinoma and cervical tumor.

In this specification, a number of documents is cited. The disclosure of these documents, including manufacturer's manuals, is herewith incorporated by reference in its entirety.

Despite intense investigations, the molecular mechanisms leading to tumor formation and cancer are far from being completely understood. Chains of molecular events leading to malignant transformation are emerging, wherein certain mechanisms appear to be generic, while others are specific for certain tumors.

Wnt and Hedgehog family proteins are secreted ligands that play multiple critical roles in the development of multicellular organisms. Alterations in the cellular signaling pathways that respond to these Wnt and Hedgehog family ligands also play causative roles in the initiation and progression of a variety of tumors (Xie and Abbruzzese (2003)). Mutations in Hedgehog signal transduction components give rise to tumors of the skin, muscle and cerebellum.

Alteration in Wingless signal transduction components is a critical step in the development of colon cancer and is associated with a variety of other types of malignancies (reviewed in Giles et al. (2003)). While alterations in the downstream components of these pathways have been known for some time, recent studies have shown that tumorigenesis can depend on the unregulated production of the ligands themselves.

One of the earliest identified oncogenes is Wnt1, whose ectopic activation in mouse mammary cells is the basis for MMTV mediated tumorigenesis (Nusse and Varmus (1992)). Consistent with this, overproduction of a variety of Wnt family ligands has been observed in human breast cancers. Over-expression of Wnt proteins in humans is also symptomatic of many gastric cancers, colorectal cancers, pancreatic cancers, esophageal cancers, squamous cell carcinomas, cervical cancers and malignant melanomas. A causative role has been directly demonstrated for Wnt5a in promoting both cell motility and invasion of malignant melanoma cells (Weeraratna et al. (2002)). Although the extent to which Wnt overproduction contributes to malignant phenotypes is not yet characterized in all cases, the frequency with which mutations in Wnt signaling pathway components promote other neoplasias suggests that Wnt overproduction has tumorigenic consequences.

Overproduction of Hedgehog ligands has been demonstrated to play a direct causal role in promoting growth of tumors of the gastrointestinal tract, including those of the esophagus, stomach biliary tract and pancreas (Berman et al. (2003); Thayer et al. (2003). These tumors are very aggressive and some of the most resistant to current therapy. The relevance of Hedgehog signalling for cancer formation and maintenance has been reviewed in Pasca di Magliano and Hebrok (2003). Preventing secretion of Hedgehog and Wnt proteins should be an effective therapy for cancers that depend on the unregulated production of these ligands.

Paradoxically, Wingless and Hedgehog are covalently modified by lipid, which is thought to mediate their interaction with the exoplasmic face of the plasma membrane. This observation raises perplexing questions about how Wingless and Hedgehog, having affinity for cell membranes, are released from the cells that make them and move through adjacent tissue. As yet, it is unclear how lipid-modified proteins leave the plasma membrane and move over many cell diameters.

The function of lipid modification of Wingless and Hedgehog is not yet understood. In *Drosophila,* mutant Hedgehog proteins that cannot be sterol-modified signal over inappropriately long distances when over-expressed (Porter et al. (1996), Burke et al. (1999)). This would suggest that the role of lipid is to restrict the range of morphogen diffusion through the epithelial plane. On the other hand, mice that harbor this mutant form of Hedgehog in its normal chromosomal context are deficient in long-range Hedgehog signalling (Lewis et al. (2001)). This suggests, in contrast, that lipid

modification may be necessary for movement of the protein. Mutations that prevent the N-terminal palmitoylation of either Wingless or Hedgehog destroy their activity (Chamoun et al. (2001), Lee et al. (2001), Willert et al. (2003)); thus, lipid modification is crucial to the function of these proteins.

In summary, there is evidence for the involvement of proteins of the Wnt or Hedgehog families in tumor formation and progression, however, a significant part of the corresponding molecular events are still obscure, thereby impeding a rational approach to therapy.

The technical problem underlying the present invention was to provide novel means and methods for the treatment of Hedgehog- and/or Wnt-secreting tumors.

Accordingly, this invention relates to the use of an inhibitor of Microsomal Triglyceride Transfer Protein (MTP), HMG-CoA reductase, DGAT and/or ACAT for the preparation of a pharmaceutical composition for the treatment of tumors.

The Microsomal Triglyceride Transfer Protein (MTP) is a heterodimeric lipid transfer protein that catalyzes the transport of triglyceride, cholesteryl ester and phosphatidylcholine between membranes. It is required for assembly and secretion of the lipoproteins containing apolipoprotein B (apoB), i.e., very low density lipoproteins (VLDL) and chylomicrons. VLDL in turn is converted into LDL. Accordingly, inhibition of MTP function would affect the levels of lipoproteins comprising chylomicrons, VLDL and LDL. Similarly, inhibitors of HMG-CoA reductase, DGAT or ACAT (which are enzymes involved in lipid biosynthesis) affect the levels of lipoproteins. The term "inhibitor" designates a compound lowering the activity of a target molecule, preferably by performing one or more of the following effects: (i) the transcription of the gene encoding the protein to be inhibited is lowered, (ii) the translation of the mRNA encoding the protein to be inhibited is lowered, (iii) the protein performs its biochemical function with lowered efficiency in presence of the inhibitor, and (iv) the protein performs its cellular function with lowered efficiency in presence of the inhibitor. In one embodiment, in particular with regard to inhibition of HMG-CoA reductase, the inhibitor is a statin. Compounds falling in class (i) include compounds interfering with the transcriptional machinery and/or its interaction with the promoter of said gene and/or with expression control elements remote from the promoter such as enhancers. Compounds of class (ii) comprise antisense constructs and constructs for performing

RNA interference well known in the art (see, e.g. Zamore (2001) or Tuschl (2001)). Compounds of class (iii) interfere with molecular function of the protein to be inhibited, in case of MTP with its enzymatic activity, in particular with the protein disulfide isomerase activity. Accordingly, active site binding compounds, in particular compounds capable of binding to the active site of any protein disulfide isomerase, are envisaged. More preferred are compounds specifically binding to an active site of MTP. Also envisaged are compounds binding to or blocking substrate binding sites of MTP as are compounds binding to or blocking binding sites of MTP for other interaction partners. An example for such an interaction partner would be apolipoproteinB (apoB). The latter group of compounds blocking binding sites of MTP may be fragments or modified fragments with improved pharmacological properties of the naturally occurring binding partners. Class (iv) includes compounds which do not necessarily directly bind to MTP, but still interfere with MTP activity, for example by binding to and/or inhibiting the function or inhibiting expression of members of a pathway which comprises MTP. These members may be either upstream or downstream of MTP within said pathway.

In a preferred embodiment, the inhibitor is a low molecular weight compound. Low molecular weight compounds are compounds of natural origin or chemically synthesized compounds, preferably with a molecular weight between 100 and 1000, more preferred between 200 and 750, and even more preferred between 300 and 600.

The efficiency of the inhibitor can be quantitized by comparing the level of activity in the presence of the inhibitor to that in the absence of the inhibitor. For example, as an activity measure may be used: the change in amount of mRNA formed, the change in amount of protein formed, the change in amount of substrate converted or product formed, and/or the change in the cellular phenotype or in the phenotype of an organism.

In a preferred embodiment, the level of activity is less than 90%, more preferred less than 80%, 70%, 60% or 50% of the activity in absence of the inhibitor. Yet more preferred are inhibitors lowering the level down to less than 25%, less than 10%, less than 5% or less than 1% of the activity in absence of the inhibitor.

Using Drosophila as a model organism, the inventors have surprisingly shown that Hedgehog and Wingless are released from cells on Lipoprotein particles. It has been

speculated that these particles (which were termed "Argosomes") represent a new type of particles. It further has been shown that these particles are required for the signaling activity of Wingless and Hedgehog (Panakova et al, in preparation). The single insect lipoprotein organized by Apolipophorin I /II is similar in biosynthesis, structure and function to vertebrate lipoprotein particles like LDL and VLDL; it transports lipid and cholesterol from the gut to the fat body (an organ analogous to the liver and adipose tissue), and distributes lipid and sterol to peripheral tissues. These similarities are evident at the sequence level as well; Apolipophorin is highly homologous to ApolipoproteinB. Therefore, drugs that inhibit the formation of lipoprotein particles in humans are expected to be useful for the treatment of tumors that secrete proteins of the Hedgehog and Wnt families.

In this regard, the Microsomal Triglyceride Transfer Protein (MTP) appears of particular interest. MTP is critical for the transfer of lipid to ApolipoproteinB and for the biosynthesis of LDL and VLDL. Compounds inhibiting MTP are known in the art and disclosed herein below. Further compounds can be identified in a straightforward manner using MTP assays described in the art. Wetterau et al. (1992) provide such an assay and a modified form has been published in Jamil et al. (1995). A new, comparably simply assay is provided in Athar et al. (2004). A further variant can be found in Gordon et al. (1996).

The data reported herein suggest a novel role for Lipoprotein particles: the transport of lipid-modified proteins. Lipid modifications like those present in Wingless, Hedgehog and gpi-anchored proteins, which target proteins to the exoplasmic membrane leaflet, would be of the correct length and topology to insert into the phospholipid monolayer of a Lipoprotein particle. The present inventors have shown that Wingless, Hedgehog and gpi-anchored proteins co-purify and co-immunoprecipitate with Drosophila Lipophorin. Futhermore, Wingless and Hedghog co-localize extensively with Lipoprotein particles in tissue. Therefore, the particles previously termed "argosomes" are actually morphogen-bearing Lipoprotein particles.

Given the data presented herein, the skilled person envisages the following further findings: (i) Reducing lipophorin abrogates Hedgehog and/or Wnt signalling; (ii) MTP activity is required for the biosynthesis of *Drosophila* lipophorin low-density particles; (iii) MTP is required for the release of Wingless and Hedgehog proteins on Lipoprotein particles; (iv) *Drosophila* MTP mutants are defective in Wingless and

Hedgehog signalling; (v) treatment with MTP inhibitors in mouse models reduces the growth of cancers, for example of the gastrointestinal tract and melanoma; and (vi) retrospective studies of patients treated for high cholesterol with MTP inhibitors show a protective effect for cancers, for example of the gastrointestinal tract and melanoma.

In supplementary experiments, the inventors have further supported their teaching that lipid-linked proteins of the exoplasmic face of the membrane associate with Lipoproteins. These include many gpi-linked proteins with diverse functions, as well as the lipid-linked morphogens Wingless and Hedgehog. The mechanism allowing long-range dispersal of lipid-linked proteins is not yet understood. The finding that these proteins exist in both membrane-associated and Lipoprotein-associated forms suggests reversible binding to Lipoprotein particles as a plausible mechanism for intercellular transfer, and the consequences of lowering lipoprotein levels in *Drosophila* larvae supports this idea.

Lipophorin knock down narrows the range of both Wingless and Hedgehog signalling. Hedgehog accumulates to an abnormally high level in cells near the source of production and long-range signaling is inhibited; short-range target genes, however, are expressed normally. These data suggest that Hedgehog does not move as far when Lipophorin levels are low. The range over which Hedgehog moves is normally restricted by Patched-mediated endocytosis. In discs from Lipophorin RNAi larvae, accumulated Hedgehog co-localizes with Patched in endosomes, suggesting that it is more efficiently sequestered by Patched.

The disclosed data is consistent with the idea that Lipophorin is continuously needed for movement, rather than required only for the release of morphogens. If Lipophorin were important only for Hedgehog secretion, one would expect Lipophorin RNAi to decrease the amount of Hedgehog found in receiving tissue; this seems not to be the case. Furthermore, altered Hedgehog trafficking in receiving tissue is consistent with a model in which Lipophorin is required at each step of intercellular transfer. Without being bound by a theory, the inventors favour the idea that reversible association of Hedgehog with Lipophorin particles facilitates its transfer from the plasma membrane of one cell to that of the next. This model predicts that lowering Lipophorin levels should increase the length of time that Hedgehog spends in the plasma membrane before becoming associated with Lipophorin. This would slow its rate of transfer and

increase the probability of Patched endocytosing Hedgehog before it moved to the next cell. Hedgehog would then signal efficiently in the short range, but be so efficiently sequestered by Patched that very little protein would travel far enough to activate long-range target genes. These predictions are completely consistent with the disclosed observations.

This model differs significantly from the original concept of argosome function. It was initially speculated that argosomes were exosome-like particles with an intact membrane bilayer, and that lipid-linked morphogens needed to be assembled on these particles to be secreted by producing cells. Instead, the present invention discloses that argosomes are exogenously derived lipoproteins that facilitate that movement of morphogens through the epithelium. Many questions remain as to how morphogens become associated with argosomes, and how the spread and cell-interactions of these particles are regulated. Clearly, heparan sulfate proteoglycans are essential for the movement of Hedgehog and Wingless into receiving tissue [35,36]. Because heparan sulfate binds to vertebrate Lipoprotein particles [37,38], one might speculate that HSPG's facilitate morphogen movement through Lipoprotein binding. Conversely, the inventors find many gpi-linked proteins, including the HSPG's Dally and Dally-like (unpublished data), on Lipoprotein particles themselves. These associated proteins have the potential to modulate the cellular affinities or trafficking properties of Lipoproteins and the morphogens they carry.

The disclosed data suggests that Lipophorin particles not only mediate intercellular transfer of Hedgehog, but may also be endocytosed together with the morphogen. Interestingly, LDL receptor related proteins Arrow and Megalin have demonstrated roles in Wingless signaling and Hedgehog endocytosis, respectively [39-41]. It is intriguing to speculate that these receptors might be important for interaction with the Lipoprotein-associated form of the morphogen.

Cholesterol has the potential to modulate the activity of the Hedgehog pathway at many different points [3,42-44]. Whether changes in the level of cellular cholesterol normally play a role in regulating the activity of the pathway is unclear. Here it is shown that Hedgehog interacts with the particle that delivers sterol to cells. This observation raises the possibility that internalization of Hedgehog is linked to sterol uptake, and suggests new mechanisms to link nutrition, growth and signalling during development. All known Hedgehog signal transduction pathways in vertebrates and

invertebrate act by regulating the processing of GLI-family transcription factors, and ectopic activation of GLI proteins is a common feature of many tumors {Ruiz i Altaba, 2002 #674}. Inhibiting the production of Lipophorin alters the processing of the drosophila GLI protein Cubitus Interruptus, causing the accumulation of an unprocessed, inactive form of the protein (Figure 21). Thus, lowering Lipophorin levels in drosophila perturbs the Hedgehog signal transduction pathway through a protein that is conserved in humans, and whose activation is characteristic of a wide variety of tumors. The present invention therefore teaches to utilize drugs that lower LDL levels in humans to treat tumors that secrete proteins of the Hedgehog and Wnt families.

Lowering Lipophorin levels also inhibits the growth of imaginal discs by mechanisms that do not depend on morphogen signalling. The inventors have shown that sterol delivery itself is essential for growth; lowering Lipophorin levels, or removing sterol from the diet blocks growth primarily at the level of cell division. Like insect cells, many human cell types do not synthesize their own sterol but rely on LDL-mediated uptake. The existence of a sterol-dependent growth control checkpoint indicates that LDL-lowering drugs should block the proliferation of tumors derived from such tissues. The inventors therefore propose to utilize drugs that lower LDL levels in humans to treat tumors derived from neurons and steroidogenic cells.

In view of the above, all steps in the life cycle of lipoproteins are envisaged as targets for therapeutic intervention. The life cycle of lipoproteins comprises formation, secretion, transport and association with a target cell. These steps are well understood and known to the skilled person. Accordingly, an alternative embodiment relates to the use of an inhibitor of lipoprotein secretion for the preparation of a pharmaceutical composition for the treatment of tumors. Another alternative embodiment relates to the use of an inhibitor of lipoprotein formation for the preparation of a pharmaceutical composition for the treatment of tumors.

Yet another alternative embodiment relates to the use of an inhibitor of lipoprotein transport for the preparation of a pharmaceutical composition for the treatment of tumors.

A further embodiment relates to the use of an inhibitor of lipoprotein association with a target cell for the preparation of a pharmaceutical composition for the treatment of tumors. Targets that affect interaction of Lipoproteins with target cells are e.g. LDL

receptors and LDL receptor family proteins, preferably LRP5 and 6, and the LRP Megalin which have been shown to be involved in wingless and hedgehog signalling. Other targets affecting interaction of Lipoproteins with target cells are Heparan sulfate proteoglycans

A further embodiment relates to the use of an inhibitor of the association of a protein of the Wnt or Hedgehog family with lipoproteins for the preparation of a pharmaceutical composition for the treatment of tumors. This class of inhibitors prevent association of Wingless or Hedgehog with Lipoproteins include any inhibitors of the acyl-transferases that acylate Wingless and Hedgehog (these are called Porcupine and Skinny Hedgehog, respectively). Another inhibitor which is in accordance with the teaching of the present invention is the enzyme acyl protein thioesterase (APT-1), which will cleave the palmitate from the Wingless protein, preventing its association with Lipoproteins (Willert et al., Nature 423, p 448).

The Wnt pathway and the Hedgehog pathways are similar, evolutionary conserved signal transduction pathways playing a role, *inter alia*, in embryogenesis and tumorigenesis. Despite identity or similarity of several components of the two pathways, the Wnt and Hedgehog protein, although located at corresponding positions of the two pathways, are unrelated proteins. The Hedgehog family of proteins includes Hedgehog (in *Drosophila*) and, in humans, Desert Hedgehog, Indian Hedgehog, and Sonic Hedgehog. The Wnt family of proteins appears to be larger, comprising the *Drosphila* proteins Wingless, DWnt2, DWnt3/5, DWnt4, DWnt6, DWnt8 and DWnt10. There are more than a dozen vertebrate Wnt proteins, from Wnt1 to Wnt16, however, not strictly sequentially numbered. Vertebrate Wnt1 is the orthologue of *Drosophila* Wingless.

In a preferred embodiment, said association of a protein of the Wnt or Hedgehog family with lipoproteins is inhibited by inhibiting the covalent lipid modification of said protein of the Wnt or Hedgehog family, thereby reducing its affinity for said lipoprotein. The lipid modifications include palmitoylation and cholesterol modification. Hedgehog proteins are palmitoylated and cholesterol modified; Wnt proteins are palmitoylated. Any inhibitor of the lipid modification of Wnt or Hedgehog proteins is an inhibitor useful for the purpose of the present invention. Accordingly, use of such inhibitors for the preparation of the pharmaceutical composition according to the invention is envisaged.

In a further preferred embodiment, said lipoprotein is very low density lipoprotein (VLDL) or low density lipoprotein (LDL).

Lipoprotein particles comprise a phospholipid monolayer surrounding a core of esterified sterol and triglycerides, and are organized by different Apolipoproteins. In vertebrates, Lipoprotein particles of different densities and apolipoprotein composition transport lipid, sterols and fat-soluble vitamins between the gut, liver and peripheral tissues. Insects have only one apolipoprotein, Apolipophorin, which is most similar to vertebrate ApoB. Physiological studies indicate that it plays a role analogous to that of vertebrate Lipoproteins, transporting sterol and diglyceride between tissues.

In a further preferred embodiment, said tumor is a malignant tumor. Malignant tumors are also referred to as cancers herein.

In a further preferred embodiment, growth and/or progression of the tumor are caused by one or more protein of the Wnt or Hedgehog family. The person skilled in the art can identify which tumors fall under this definition without undue burden.

In a further preferred embodiment, said tumor over-expresses one or more protein of the Wnt or Hedgehog family. The skilled person is aware of concrete tumors and malignant diseases comprised in this definition. Furthermore, means and methods are described below enabling the person skilled in the art to identify which tumors or malignant diseases are encompassed by the embodiment recited above.

The term "over-expression" denotes an expression level of an mRNA encoding a protein of the Wnt or Hedgehog family and/or of a protein of the Wnt or Hedgehog family, which is elevated in comparison to normal expression. The term "normal expression" refers to a reference expression level determined in one or more samples from healthy individuals. These samples are preferably from healthy tissue corresponding to the tissue affected by the tumor under consideration. Samples may be drawn from a mixed population, from a fraction of the population, wherein the population has previously been stratified according to one or more parameters, or from healthy regions of the tissue affected by the tumor from the same patient. Statistical methods known in the art may be used in order to assign significance values and confidence intervals to the measured expression and over-expression data.

In a more preferred embodiment, the expression level to be determined is the mRNA

expression level. Methods for the determination of mRNA expression levels are known in the art and comprise Real Time PCR, Northern blotting and hybridization on microarrays or DNA chips equipped with one or more probes or probe sets specific for transcripts encoding proteins of the Wnt or Hedgehog family.

In another more preferred embodiment, the expression level to be determined is the protein expression level. The skilled person is aware of methods for the quantitation of proteins. Amounts of purified protein in solution can be determined by physical methods, e.g. photometry. Methods of quantifying a particular protein in a mixture rely on specific binding, e.g of antibodies. Specific detection and quantitation methods exploiting the specificity of antibodies comprise immunohistochemistry (*in situ*) and surface plasmon resonance. Western blotting combines separation of a mixture of proteins by electrophoresis and specific detection with antibodies.

In a further preferred embodiment, said protein of the Wnt family is Wnt1, Wnt2, Wnt2B, Wnt3, Wnt3A, Wnt4, Wnt5A, Wnt5B, Wnt6, Wnt7A, Wnt7B, Wnt8A, Wnt8B, Wn9A, Wnt9B, Wnt10A, Wnt10B, Wnt11 and/or Wnt16 and/or said protein of the Hedgehog family is Desert Hedeghog, Indian Hedgehog and/or Sonic Hedgehog. A list of Wnt genes and pertinent information is maintained by Roel Nusse at http://www.stanford.edu/~rnusse/wntwindow.html.

In a further preferred embodiment, said tumor is selected from the group consisting of esophageal tumor, biliary tract tumor, gastric tumor, pancreatic tumor and malignant melanoma. For these tumors, Wnt and/or Hedgehog family proteins have been shown to play a causative role in tumor growth or progression.

Yet a further preferred embodiment relates to a tumor selected from the group consisting of gastric tumor, colorectal tumor, pancreatic tumor, esophageal tumor, squamous cell carcinoma, cervical tumor and malignant melanoma. These tumors have been shown to over-express one or more members of the Wnt and/or Hedgehog families of proteins.

In a further preferred embodiment, the tumor or cancer is a tumor or cancer of muscle, cerebellum or breast.

One class of drugs known to reduce Lipoprotein secretion acts by inhibiting the activity of Microsomal Triglyceride Transfer Protein (MTP). MTP is critical for the

transfer of lipid to ApolipoproteinB and for the biosynthesis of LDL and VLDL. The Drosophila MTP homologue has a conserved enzymatic activity and can support the secretion of ApoB-containing Lipoprotein. A spectrum of small molecule MTP inhibitors is already used in patients to treat elevated serum cholesterol. MTP inhibitors and other inhibitors of LDL secretion are expected to be effective against Hedgehog and Wnt-secreting tumors.

Other classes of drugs known to reduce Lipoprotein secretion act by inhibiting the activity of HMG-CoA reductase (statins), diacylglycerol acyl transferase and ACAT (acyl-CoA:cholesterol acyl-transferase). ACAT inhibitors include CI-1-11, FCE 27677 (Musanti et al., 1996), DuP 128 (Higley et al., 1994), CI-976 (Krause et al., 1993), PD-138142-15, CP-113818 (Marzetta et al.,1994), HL-004 (Murakami et al., 1995), FR-145237 (Matsuo et al., 1995), TMP-153 (chemical formula = N-[4-(2-chlorophenyl)-6,7-dimethyl-3-quinolyl]-N'-(2,4-difluorophe nyl)urea. HMG-CoA reductase inhibitors include e.g.atorvastatin (lipitor) (Topliss et al., 2002), simvastatin (Topliss et al. 2002), lovastatin (Topliss et al. 2002), cerivastatin (Yasunobu et al., 1997), pravastatin (BMS product info sheet), fluvastatin (Suzumura et al., 1999), mevastatin (compactin) (Chakravarti et al., 2004), rosuvastatin (Bolego et al., 2002), pitavastatin (Bolego et al., 2002. Diacylglycerol acyl transferase -1 inhibitors include Gemfibrozil: 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (Ammazzalorso et al., 2002; Zhu et al., 2002).

Accordingly, in a preferred embodiment, said inhibitor of MTP is selected from the group consisting of diaminoindanes (derivatives of indane) such as 8aR or 19aR (Ksander et al. (2001)); citrus flavonoids (derivatives of flavone) such as naringenin (Borredaile et al. (2003)) or hesperetin; quercitin (Casaschi et al. (2002)); BMS-197636 (Harrity et al. (1996)); BMS-201038 (Wetterau et al. (1998)); BMS-200150 (Jamil et al. (1996)); other benzimidazole-based MTP inhibitors (derivatives of benzimidazole) (Robl et al. (2001)); CP-346086 (Chandler et al. (2003)) and Implitapide (BAY-13-9952) (Shiomi and Ito (2001)). Compounds belonging to classes such as diaminoindanes, flavonoids and benzimidazoles mentioned above, wherein said compounds are not explicitly disclosed herein or in the references cited herein are also envisaged. Assays suitable for identifying compounds belonging to these classes and capable of inhibiting MTP are known to the skilled person and can be found in references cited herein above. In another preferred embodiment of the present invention, said inhibitor of ACAT is selected from the group consisting of CI-

1-11, FCE 27677, DuP 128, CI-976, PD-138142-15, CP-113818, HL-004, FR-145237, TMP-153 (chemical formula = N-[4-(2-chlorophenyl)-6,7-dimethyl-3-quinolyl]-N'-(2,4-difluorophe nyl)urea. In another preferred embodiment of the present invention, said inhibitor of HMG-CoA is selected from atorvastatin (lipitor), simvastatin, lovastatin, cerivastatin, pravastatin, fluvastatin, mevastatin (compactin), rosuvastatin, pitavastatin. In another preferred embodiment of the present invention, the Diacylglycerol acyl transferase -1 inhibitor is Gemfibrozil: 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid.

The pharmaceutical compositions prepared according to the invention may further comprise a pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 µg; however, doses below or above this exemplary range are envisioned as well. Preferably, the dose is in the range of 0.01 to 100 µg, more preferred between 0.1 and 10 µg. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. The compositions prepared according to the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl

oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use of the pharmaceutical composition.

In a preferred embodiment of the present invention, the lipoprotein inhibitor is selected from siRNA, shRNA, ribozyme, antisense nucleic acid molecule, aptamer or inhibitory antibody.

In a more preferred embodiment of the present invention, the siRNA, shRNA, ribozyme and antisense nucleic acid molecules have a region of complementarity with at least one gene encoding a protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell. Said target may, for example, be selected from the group consisting of MTP, diacylglycerol acyltransferase, or enzymes in the cholesterol biosynthesis pathway such as HMGCoA reductase.

Techniques to generate functional siRNA constructs against given target sequences are well known in the art and are referred to herein. The target sequence of the ACAT gene (acyl-CoA:cholesterol acyl-transferase) may be any sequence selected from:

CTAAAGCGAGAACTGTCGCCCTTCACGATGTGGCTCCGTGCCTTTATCCTGGCC
ACTCTCTCTGCTTCCGCGGCTTGGGGGGCACATCCGTCCTCGCCACCTGTGGT
GGACACCGTGCATGGCAAAGTGCTGGGGAAGTTCGTCAGCTTAGAAGGATTTG
CACAGCCTGTGGCCATTTTCCTGGGAATCCCTTTTGCCAAGCCGCCTCTTGGAC
CCCTGAGGTTTACTCCACCGCAGCCTGCAGAACCATGGAGCTTTGTGAAGAATG
CCACCTCGTACCCTCCTATGTGCACCCAAGATCCCAAGGCGGGGCAGTTACTCT
CAGAGCTATTTACAAACCGAAAGGAGAACATTCCTCTCAAGCTTTCTGAAGACTG
TCTTTACCTCAATATTTACACTCCTGCTGACTTGACCAAGAAAAACAGGCTGCCG
GTGATGGTGTGGATCCACGGAGGGGGGCTGATGGTGGGTGCGGCATCAACCT

ATGATGGGCTGGCCCTTGCTGCCCATGAAAACGTGGTGGTGGTGACCATTCAAT
ATCGCCTGGGCATCTGGGGATTCTTCAGCACAGGGGATGAACACAGCCGGGGGG
AACTGGGGTCACCTGGACCAGGTGGCTGCCCTGCGCTGGGTCCAGGACAACAT
TGCCAGCTTTGGAGGGAACCCAGGCTCTGTGACCATCTTTGGAGAGTCAGCGG
GAGGAGAAAGTGTCTCTGTTCTTGTTTTGTCTCCATTGGCCAAGAACCTCTTCCA
CCGGGCCATTTCTGAGAGTGGCGTGGCCCTCACTTCTGTTCTGGTGAAGAAAG
GTGATGTCAAGCCCTTGGCTGAGCAAATTGCTATCACTGCTGGGTGCAAAACCA
CCACCTCTGCTGTCATGGTTCACTGCCTGCGACAGAAGACGGAAGAGGAGCTC
TTGGAGACGACATTGAAAATGAAATTCTTATCTCTGGACTTACAGGGAGACCCC
AGAGAGAGTCAACCCCTTCTGGGCACTGTGATTGATGGGATGCTGCTGCTGAAA
ACACCTGAAGAGCTTCAAGCTGAAAGGAATTTCCACACTGTCCCCTACATGGTC
GGAATTAACAAGCAGGAGTTTGGCTGGTTGATTCCAATGCAGTTGATGAGCTAT
CCACTCTCCGAAGGGCAACTGGACCAGAAGACAGCCATGTCACTCCTGTGGAA
GTCCTATCCCCTTGTTTGCATTGCTAAGGAACTGATTCCAGAAGCCACTGAGAA
ATACTTAGGAGGAACAGACGACACTGTCAAAAAGAAAGACCTGTTCCTGGACTT
GATAGCAGATGTGATGTTTGGTGTCCCATCTGTGATTGTGGCCCGGAACCACAG
AGATGCTGGAGCACCCACCTACATGTATGAGTTTCAGTACCGTCCAAGCTTCTC
ATCAGACATGAAACCCAAGACGGTGATAGGAGACCACGGGGATGAGCTCTTCT
CCGTCTTTGGGGCCCCATTTTTAAAAGAGGGTGCCTCAGAAGAGGAGATCAGAC
TTAGCAAGATGGTGATGAAATTCTGGGCCAACTTTGCTCGCAATGGAAACCCCA
ATGGGGAAGGGCTGCCCCACTGGCCAGAGTACAACCAGAAGGAAGGGTATCTG
CAGATTGGTGCCAACACCCAGGCGGCCCAGAAGCTGAAGGACAAAGAAGTAGC
TTTCTGGACCAACCTCTTTGCCAAGAAGGCAGTGGAGAAGCCACCCCAGACAG
AACACATAGAGCTGTGAATGAAGATCCAGCCGGCCTTGGGAGCCTGGAGG

The target sequence of the ACAT2 gene (acyl-CoA:cholesterol acyl-transferase 2) may be any sequence selected from:

AAAAATTACAAAAATTAGCCAGATGTGGTGGCATGCACCTGTAATCCCAGCTACT
CAGGAGGCTGAGGCAAGAGAATTGCTTGAACCTGGGAGGTGGAGGTTGCAGTG
AGCAGAGATCATGCCAATGCATTCCAGCCAGGGCGACAGAGCGAGACTCCATC
TCAAAAAAAAAAAAAAAAAAAAAAGAGAGGGAACCCCCGAGGAGACGCTCAGGT
GTTTTTACATCCTCAGACTCAGATGTCCCCTCTCATTCTCCTGCTGGAAGCCTCT
GGCCTCACTCCTGACCTGTGTGCCTCTCTCTCCAGTGCCGCTCCTCCCAGGGG
CAGCGCCGAGGTCCCTGCTGGTGTGTGGATCGGATGGGCAAGTCCCTGCCAG

GGTCTCCAGATGGCAATGGAAGCTCCTCCTGCCCCACTGGGAGTAGCGGCTAA
AGCTGGGGGATAGAGGGGCTGCAGGGCCACTGGAAGGAACATGGAGCTGTCA
TCACTCAATAAAAAACCGAGGCCCTCAATCCACCTTCAGGCCCCGCCCCATGGG
CCCCTCACCGCTGGTTGGAAAGAGTGTTGGTGTTGGCTGGGGTGTCAATAAAG
CTGTGCTCGGGGTCGCTGGCTTGTGTCTCTGTGTCTGCCTCTCACAATTCTGGA
ATCCCTGGCCCTCTCTTTACCCCACTACAGCTCACTCACAGCATTTCTCCTTTCC
TGTGGATACCTTTAGTCCTTCCTGTGACAGCCAGGCAGAAGCTTCAAGCCATCA
AGCCTCACAGAGCTGCCCACCAGGAGTTGGGAGTGGGAAGGGGAGACACTGA
GATCATGGCCTTAATCTGAAGTTGTGACCTTGTTTCTAACACTGATCTTTGGACA
CAAAGGGAGGGGAAGGATTAATAGTTAATCCCAGCAGGAACCCAGCAAAGAGG
AACTCTCAAAGCACATACCCTTCTGTTACTTCCTACTAAAAAAAGAAGGAAATTAT
TACTAATATATGAGCTCATCCCATGGCCCTGAACCATGTGATTTTACCTGGACAA
CCTCATTTTGAGCTTACGATAACCTTGTGATATAGGGATTTTTACCCCTATTTTTC
AGAGGAGGAAACTGGCTTAAAATTTGGGGTCACTTGCCTGAAATTATATAGCTG
GTAAATGACAGAGGGAGGTTTGCATCCGGTTCTTCCATATGTGACAATACCTGG
TATTCCCTAGGTGCTGAATACGTGTTTGTTTAGTCTCCTTTCTGCCTACATGCTC
ACCCAAGCAGGTGTCAGGAAGCGGCCCTGTCAGTTCAGGGGCCCTGACACTCA
GCCTTTCCTGGAGGGGCCCCAGTTCCGTGAGTAGCACAGTGCCAACCCCATCA
GAATATTACCACATGTGTCAATCACACACCTTGTGGTGAAAGCGAGCTGAACGC
ACTGATACATGAAGACATTTCTGACTCCTCCCTGACCTTCAGCCTGCTGGGAGA
GAGACTGGGAGGCCAGCCATGCTAGGTGACAAGCTTCTGAGAGGCAAAGTTCC
CCTCCCCAAGATGTACCCAGCCACTATTCCTGTGTGTGTGTGGCGGGGTGGAT
AGCACCCTTGGAGCTGGGCATCTGGATGGTGGTTGGGGTGTTAGGGGGATGTG
GCGATCCCTGCGACAGACAGCAGAGTGAGGTCTGAATCTGAGAAGCTCTGCCT
CCAGATCAGATAACCTATCGCACTCCCAGAGGCCCCCTCCTCCTGAAAGGACTT
TAGTCTTTGGAGCTGTCACCTGAGCTGAGTGGGACAAGAGCTCTACAGGGCAG
GCCACACTGCGAAGGAAGGAGGCAACACGGGCAAGGGCTGCCTGCTGCCCGC
TGGAGACCGCACCATGGAGCCAGGCGGGGCCCGTCTGCGTCTGCAGAGGACA
GAAGGGCTGGGAGGGGAGCGGGAGCCCCAACCCTGTGGAGATGGTGAGCCG
CCCTCGGGGGTTGCAATAAGGCACAGTGCAAGTTGGGGGGGAGGCGGGGAGA
GATGCGCTATGGAGAGAAGGCTCCAACTGCCTGATGCCAATCCTTCCTGCTGTC
CAGAGAGGCCAAGGAAGGTAGACCCTGGCCTCAGCCCAGAGCCACCCAGCTG
GAAGATGGAACAGAAAAGAGCTATGTCAGAGCTGTGGCCTGGCCTTATCCTTCT
GACTGTTTAGCCCCAGATAGTGAATGGAGTAGCTTCCCATTCCCTGAGTGCCCT

GTTCCCAGTAATGTCACCAGGCCCCCTGCTTGCCTATGGCCTCCTCACCACTGG
CAACAGCAACTTCCCCTTCTAGTAGCCCCAACCATGATACTAGATATTGGCTGG
TTGGGGTGAGGGCAGCTGCTGAGCACACAAAGCATTTCCTGACCACAGGATGC
CTCTGGGTCTGCAGAACCCCAATTCCTCCTGTTGACTGTGCCTTTGATCCCTCC
TCACAGGAAACACTGAGACGCACAGAGCCCCGGACTTGGTACAATGGACCCGA
CACATGGAGGTGAGGGATGTCAGAGGTCAGAGGTCAAGTGGACAGATCCTTGG
TAGCAGGAAGCAGGAGTGAGGCTTGGTGATAAAGATGACTTTTTTTTTTTTTTT
TTTTGAAACAAAGTCTCGCTCTGTTGCTCAGGCTGGAGTGCATTGGCACAATCT
CGGCTCACTGCAACCTCTGCCTCCCAGGTTCAAGCGAGTAGCTGAACTACAGAT
GAGTGCCACCAGGTGTGGCTAATTTTTTTTTTATATTTTTAGTAGAGACGGGGTT
TCACCATATTGGCCAGGCTGGTCTTGAACTCCTGACCTCAAGTGATCTACCCGC
CTCGGCCTCCCAAAGTGCTGGGATTACAGGTGTGAGCCACGGTGCCCAGCTTT
GATGACTTGTTCCTGAGTGCAAAGATCGGATTCGAATTAAGGATGGAGATGGAG
GGGTTTGAAGCACAGGTCCCAAAGGTCACCAAAAGTCAGCCAGTAGATAGAGT
CTTCCTGCTAGTGCGGCTGGAGGCCAGGGGTGAGGAGTGACAATCGGAGGTG
ACCAGGCTGGAATTCAGACCCAGGGAGGGGAAGCCTTGCTCCTGCTGTCACTC
CCTGATCCTCGCTGCTGCCCCAGGAAGCCAGAAGGAGATCAACTCTGGGCAGG
GATCATGGCTTGTTCATCTTTTTACCACAGCACTTAGCACTGCGGGGTCCTACC
CCACCAGCAGACCCATCACCACCCCACCAATGCCTGCCCTTCCCCCCGCAGGC
CTGCCACCACTGAACCCCCATCCCTGCTGACCCCCCAGGTTGTTTTTTTTTTTAA
AAAAAGCACTGTGCCTGGCATAGACAGAAATTCAGTGAATGTCTGGTGAATGAA
AGGATGGCTGACTGGAGGGACAGTGGGCTCTACCCTGGCTTTGTCCCGTAGGC
TGTGAAGGCACAATTGCTGGAGCAAGCGCAGGGACAACTGAGGGAGCTGCTGG
ATCGGGCCATGCGGGAGGCTATACAATCCTACCCATCACAAGACAAACCTCTGC
CCCCACCTCCCCCAGGTTCCTTGAGCAGGTGAGTCTGGGGAATGGCTGCGCGG
GCTCCTCTGTAGCAAGGATCATGAGCTAGAAACATCACGTTATGTGCCCTTGCC
AGCTTTATCAGGACCGTGACTCCAGCCTTCCCTCTTCCCCCCTTGTCTTCCTAAC
ACTGACCTCCATCTACTGGGCCTCGCTCTGCCCTGGCCCTGACCTTCTACCCG
GTCCTGCCCTCTGGCCCTAGCCCTGACCTTTCCCTACCACTCCCCTTGGCTCCC
AAGTATTGACCTTCTGCATGGCCCTGCCCTCTGCCCATTTACTTTTTCCTGACCT
TGAACAAACATCTCAATTCAGGACCCAGGAGCCATCCCTGGGGAAACAGAAAGT
TTTCATCATCCGCAAGTCCCTGCTTGAGTAAGTCGGGGTGATGACAAGTAATGG
GAGGAAAAGAAAGGGCTTTGGCTAGGGGTCAAGGTACCTCATTTCTCATCCCCA
GCCTCTAGGTGGTTTGTGGAGAGGACTTGGAAAGGGTTTCTAAGGTGCAGGTC

CCCTATTTGCCCTAGGCATGGTTGTGGAAGAAAGGCCTTTAGCTGAAGTTCTGG
ATCGCTAGGCCCCATGCCTCATGTGTACAGTGTCTGAGCTAGTTCACACAACCC
TGAGGACCCTCCCACACTGACTTTCGGGCCCCTGTCCCTCTAGTGAGCTGATG
GAGGTGCAGCATTTCCGCACCATCTACCACATGTTCATCGCTGGCCTGTGTGTC
TTCATCATCAGCACCCTGGCCATCGACTTCATTGATGAGGGCAGGTAGGTCCCC
TTCCCACCTGGGACAGGCACACCTATCTGATCAGACCCTCTGGGTCCTCAGTGC
CCCACCTGCCCTTGGGGCAAGAGGACACAGGTTCCCCCTTTGGTTATTGGACAT
GTACCAACATTGCTCCCAACAACCTTTCCACCTGAAACACTCTCTAGGATCCCTT
CCTTCCCCAGCCTTGGTTGGCCCTTTGTCTGTCCTCCCAGGGCCGGCCACTCT
CAAATGCTTCTCAATGACACCTTGCCCTGAGGCAGATGTGTGTGTGTGTGCACA
CATGCACACCCATACACACTCCTTTCCAAGTCAGTTCTATTCAAGTAACCTCCAT
TGAGCACCTCCCGAGTACCAGTCCTAGTGGCCAGAGAAATGAGGGGGGCAGAC
CCCACTCCTGTCTGTGAGGCACTCCCAGTCTTGTGGGGAAGACAGACATGTAAC
CAAGTGCCTGGGTGGGTCATTTCCCCCACAGAGCACCCCATCCAACTGTGAGG
ATGAGCCTCAGAGAGCTGTATACCCCTGGGGGTCTGTATTTTCATACCTCCCAC
TATTCCCAGACCAAACTGAGGGTTGGGGCTGCTATTTCTCATGGCCCAATAACG
AGATGCAGATGAACTGGGGAGGAAGTGAGTTTTTATTTCTGTAACCAGTTACAG
GGAGAAGGCCTGGAAATTATCACCAGGCCAACTAAAAATTACAATTTCCAGAGC
ATATATACCTTCTAAGCTCTACATCTATGTGTAGGTGTGCATTCATCTAAAGACAT
AAGTGATTAACTTCTTTTAATCTATAACTAAGGTCTGAGTCTTGAAGACCTTCTTC
TGGAGCCTCAGTAAGTTTACTTAATCTAAATGGGTCCAAGTGCTGGGGTGATTA
CCCTTCTCTTATCTCCTGCTAAATAACGGAGGTTTGGGGAGTTTCTTCAGACCCC
CAGCAAAACTTGTTTAATCATGCTTTGAGGTTCAGGAAAGGCCTAGGCAAAACT
CTTGGTGGGCTTTTGTTACATTCCAGCCTTTGTATAAGGGCACTGGTTTTTAACA
TTTAACTTAACCACTCAGTCAGTACTGAAACAGTTGTTATGGAGGTCTGCATTAG
TGAGACCTGCTTGTCACAGTCCCCACTGTCAATTTACACGATTTTTATCATGCAT
GGATATTTATTTATCATGAGAATCGCAGGGAGATGGGGAGTCGTAATCTTTCTG
GCTACTTCCTGCTGAGAGGGGGTCGTCGTTATGGGGCACCAAAAGCAGCAGTG
GAGTGGAAGAGGTTGATTTGTTCGCAGTAGCACTCTCTGTTTCGGGGCCTTGGA
GGCAGCACCTGCTGAAACATAATAGTATGTAACAGTACATATAAATAGGCTACTG
CTCTTTTCTTCTGAAGTTTAAGTTGTCTAGTCTTCAGTTTGCAGGGCTTTAAGAAA
GCAGAGCTTAGGTTTCAGTGATTACCAATTAGGAAGAATGGGGGAAAATGGAAA
AGAAAGAGGAAAAAATTGAAAACATTATTTTGGAGACCTGTAGCCAGAAAAGTTA
GAATTTAATCCAAACTGCAGAAAATAATAAAAACTGAAAAACATCAGGCAAGACT

AGAATTTAACAACAGATGTACTTTTTTTTTTTTTTTTTGAGACAGAGTCTTGGTC
CGTTGCCCAGGCTGGAGTGCAGTGGCGTGATCTTGGCTCACTGCAACCTCCGC
CTCCTGGGTTCAAGCGAATCTCCAACCTCAGCCTCCTGAGTAGCTGGGACTACA
GGCACGTGTTACTGTGTTCGGCTCATTTTTGTATTTTTAGTAGAGACTGGGTTTC
ACCGTGTTGGCCAGGCTGGTCTCGAACTCCTGACCTCAGGCGATCTGCCCACC
TTGGCCTCCCAAAGTGCTGGGATTACAGGTGTGAGCCACTGAGCCCAGCCATA
GTTTTTGAAACATAATTTTTCTCTCTCCAGTTTCCCATTTTTATTAAAAGACAGATC
ATAGTAGGACTGGTTTGCTTTATTATACTTGGTTTAATTATTTGTATACAGTACAG
CAAGAATAATTAGTTTCACATAGGCCTTTTAAATTGGCTTTGATGGAACTTCGTTC
CATAGAAGGAATCTGAGATAAGTCCTTTTAAAGCCGAGCCCCAGCCATGGATTT
GTACCAATAAATACCTATGAGTTGGGTGAATTCCTCTTCTCTTGA

The target sequence of the diacylglycerol acyl transferase gene (DGAT1) may be any sequence selected from:

GAATGGACGAGAGAGGCGGCCGTCCATTAGTTAGCGGCTCCGGAGCAACGCA
GCCGTTGTCCTTGAGGCCGACGGGCCTGACGCGGGCGGGTTGAACGCGCTGG
TGAGGCGGTCACCCGGGCTACGGCGGCCGGCAGGGGGCAGTGGCGGCCGTT
GTCTAGGGCCCGGAGGTGGGGCCGCGCGCCTCGGGCGCTACGAACCCGGCA
GGCCCACGCTTGGCTGCGGCCGGGTGCGGGCTGAGGCCATGGGCGACCGCG
GCAGCTCCCGGCGCCGGAGGACAGGGTCGCGGCCCTCGAGCCACGGCGGCG
GCGGGCCTGCGGCGGCGGAAGAAGAGGTGCGGGACGCCGCTGCGGGCCCCG
ACGTGGGAGCCGCGGGGGACGCGCCAGCCCCGGCCCCCAACAAGGACGGAG
ACGCCGGCGTGGGCAGCGGCCACTGGGAGCTGAGGTGCCATCGCCTGCAGGA
TTCTTTATTCAGCTCTGACAGTGGCTTCAGCAACTACCGTGGCATCCTGAACTG
GTGTGTGGTGATGCTGATCTTGAGCAATGCCCGGTTATTTCTGGAGAACCTCAT
CAAGTATGGCATCCTGGTGGACCCCATCCAGGTGGTTTCTCTGTTCCTGAAGGA
TCCCCATAGCTGGCCCGCCCCATGCCTGGTTATTGCGGCCAATGTCTTTGCTGT
GGCTGCATTCCAGGTTGAGAAGCGCCTGGCGGTGGGTGCCCTGACGGAGCAG
GCGGGACTGCTGCTGCACGTAGCCAACCTGGCCACCATTCTGTGTTTCCCAGC
GGCTGTGGTCTTACTGGTTGAGTCTATCACTCCAGTGGGCTCCCTGCTGGCGCT
GATGGCGCACACCATCCTCTTCCTCAAGCTCTTCTCCTACCGCGACGTCAACTC
ATGGTGCCGCAGGGCCAGGGCCAAGGCTGCCTCTGCAGGGAAGAAGGCCAGC
AGTGCTGCTGCCCCGCACACCGTGAGCTACCCGGACAATCTGACCTACCGCGA
TCTCTACTACTTCCTCTTCGCCCCCACCTTGTGCTACGAGCTCAACTTTCCCCGC
TCTCCCCGCATCCGGAAGCGCTTTCTGCTGCGACGGATCCTTGAGATGCTGTTC

TTCACCCAGCTCCAGGTGGGGCTGATCCAGCAGTGGATGGTCCCCACCATCCA
GAACTCCATGAAGCCCTTCAAGGACATGGACTACTCACGCATCATCGAGCGCCT
CCTGAAGCTGGCGGTCCCCAATCACCTCATCTGGCTCATCTTCTTCTACTGGCT
CTTCCACTCCTGCCTGAATGCCGTGGCTGAGCTCATGCAGTTTGGAGACCGGG
AGTTCTACCGGGACTGGTGGAACTCCGAGTCTGTCACCTACTTCTGGCAGAACT
GGAACATCCCTGTGCACAAGTGGTGCATCAGACACTTCTACAAGCCCATGCTTC
GACGGGGCAGCAGCAAGTGGATGGCCAGGACAGGGGTGTTCCTGGCCTCGGC
TTTCTTCCACGAGTACCTGGTGAGCGTCCCTCTGCGAATGTTCCGCCTCTGGGC
TTTCACGGGCATGATGGCTCAGATCCCACTGGCCTGGTTCGTGGGCCGCTTTTT
CCAGGGCAACTATGGCAACGCAGCTGTGTGGCTGTCGCTCATCATCGGACAGC
CAATAGCCGTCCTCATGTACGTCCACGACTACTACGTGCTCAACTATGAGGCCC
CAGCGGCAGAGGCCTGAGCTGCACCTGAGGGCCTGGCTTCTCACTGCCACCTC
AAACCCGCTGCCAGAGCCCACCTCTCCTCCTAGGCCTCGAGTGCTGGGGATGG
GCCTGGCTGCACAGCATCCTCCTCTGGTCCCAGGGAGGCCTCTCTGCCCTATG
GGGCTCTGTCCTGCACCCCTCAGGGATGGCGACAGCAGGCCAGACACAGTCTG
ATGCCAGCTGGGAGTCTTGCTGACCCTGCCCCGGGTCCGAGGGTGTCAATAAA
GTGCTGTCCAGTGGGAG

The target sequence of the HMGCoA reductase gene may be any sequence selected from:

TTCGGTGGCCTCTAGTGAGATCTGGAGGATCCAAGGATTCTGTAGCTACAATGT
TGTCAAGACTTTTTCGAATGCATGGCCTCTTTGTGGCCTCCCATCCCTGGGAAG
TCATAGTGGGGACAGTGACACTGACCATCTGCATGATGTCCATGAACATGTTTA
CTGGTAACAATAAGATCTGTGGTTGGAATTATGAATGTCCAAAGTTTGAAGAGGA
TGTTTTGAGCAGTGACATTATAATTCTGACAATAACACGATGCATAGCCATCCTG
TATATTTACTTCCAGTTCCAGAATTTACGTCAACTTGGATCAAAATATATTTTGGG
TATTGCTGGCCTTTTCACAATTTTCTCAAGTTTTGTATTCAGTACAGTTGTCATTC
ACTTCTTAGACAAAGAATTGACAGGCTTGAATGAAGCTTTGCCCTTTTTCCTACT
TTTGATTGACCTTTCCAGAGCAAGCACATTAGCAAAGTTTGCCCTCAGTTCCAAC
TCACAGGATGAAGTAAGGGAAAATATTGCTCGTGGAATGGCAATTTTAGGTCCT
ACGTTTACCCTCGATGCTCTTGTTGAATGTCTTGTGATTGGAGTTGGTACCATGT
CAGGGGTACGTCAGCTTGAAATTATGTGCTGCTTTGGCTGCATGTCAGTTCTTG
CCAACTACTTCGTGTTCATGACTTTCTTCCCAGCTTGTGTGTCCTTGGTATTAGA

GCTTTCTCGGGAAAGCCGCGAGGGTCGTCCAATTTGGCAGCTCAGCCATTTTG
CCCGAGTTTTAGAAGAAGAAGAAAATAAGCCGAATCCTGTAACTCAGAGGGTCA
AGATGATTATGTCTCTAGGCTTGGTTCTTGTTCATGCTCACAGTCGCTGGATAGC
TGATCCTTCTCCTCAAAACAGTACAGCAGATACTTCTAAGGTTTCATTAGGACTG
GATGAAAATGTGTCCAAGAGAATTGAACCAAGTGTTTCCCTCTGGCAGTTTTATC
TCTCTAAAATGATCAGCATGGATATTGAACAAGTTATTACCCTAAGTTTAGCTCTC
CTTCTGGCTGTCAAGTACATCTTCTTTGAACAAACAGAGACAGAATCTACACTCT
CATTAAAAAACCCTATCACATCTCCTGTAGTGACACAAAAGAAAGTCCCAGACAA
TTGTTGTAGACGTGAACCTATGCTGGTCAGAAATAACCAGAAATGTGATTCAGTA
GAGGAAGAGACAGGGATAAACCGAGAAAGAAAAGTTGAGGTTATAAAACCCTTA
GTGGCTGAAACAGATACCCCAAACAGAGCTACATTTGTGGTTGGTAACTCCTCC
TTACTCGATACTTCATCAGTACTGGTGACACAGGAACCTGAAATTGAACTTCCCA
GGGAACCTCGGCCTAATGAAGAATGTCTACAGATACTTGGGAATGCAGAGAAAG
GTGCAAAATTCCTTAGTGATGCTGAGATCATCCAGTTAGTCAATGCTAAGCATAT
CCCAGCCTACAAGTTGGAAACTCTGATGGAAACTCATGAGCGTGGTGTATCTAT
TCGCCGACAGTTACTTTCCAAGAAGCTTTCAGAACCTTCTTCTCTCCAGTACCTA
CCTTACAGGGATTATAATTACTCCTTGGTGATGGGAGCTTGTTGTGAGAATGTTA
TTGGATATATGCCCATCCCTGTTGGAGTGGCAGGACCCCTTTGCTTAGATGAAA
AAGAATTTCAGGTTCCAATGGCAACAACAGAAGGTTGTCTTGTGGCCAGCACCA
ATAGAGGCTGCAGAGCAATAGGTCTTGGTGGAGGTGCCAGCAGCCGAGTCCTT
GCAGATGGGATGACTCGTGGCCCAGTTGTGCGTCTTCCACGTGCTTGTGACTCT
GCAGAAGTGAAAGCCTGGCTCGAAACATCTGAAGGGTTCGCAGTGATAAAGGA
GGCATTTGACAGCACTAGCAGATTTGCACGTCTACAGAAACTTCATACAAGTATA
GCTGGACGCAACCTTTATATCCGTTTCCAGTCCAGGTCAGGGGATGCCATGGG
GATGAACATGATTTCAAAGGGTACAGAGAAAGCACTTTCAAAACTTCACGAGTAT
TTCCCTGAAATGCAGATTCTAGCCGTTAGTGGTAACTATTGTACTGACAAGAAAC
CTGCTGCTATAAATTGGATAGAGGGAAGAGGAAAATCTGTTGTTTGTGAAGCTG
TCATTCCAGCCAAGGTTGTCAGAGAAGTATTAAAGACTACCACAGAGGCTATGA
TTGAGGTCAACATTAACAAGAATTTAGTGGGCTCTGCCATGGCTGGGAGCATAG
GAGGCTACAACGCCCATGCAGCAAACATTGTCACCGCCATCTACATTGCCTGTG
GACAGGATGCAGCACAGAATGTTGGTAGTTCAAACTGTATTACTTTAATGGAAG
CAAGTGGTCCCACAAATGAAGATTTATATATCAGCTGCACCATGCCATCTATAGA
GATAGGAACGGTGGGTGGTGGGACCAACCTACTACCTCAGCAAGCCTGTTTGC
AGATGCTAGGTGTTCAAGGAGCATGCAAAGATAATCCTGGGGAAAATGCCCGG

CAGCTTGCCCGAATTGTGTGTGGGACCGTAATGGCTGGGGAATTGTCACTTATG
GCAGCATTGGCAGCAGGACATCTTGTCAAAAGTCACATGATTCACAACAGGTCG
AAGATCAATTTACAAGACCTCCAAGGAGCTTGCACCAAGAAGACAGCCTGAATA
GCCCGACAGTTCTGAACTGGAACATGGGCATTGGGTTCTAAAGGACTAACATAA
AATCTGTGAATTAAAAAAGCTCAATGCATTGTCTTGTGGAGGATGAATAAATGTG
ATCACTGAGACAGCCACTTGGTTTTTGGCTCTTTCAGAGAGGTCTCAGGTTCTTT
CCATGCAGACTCCTCAGATCTGAACACAGTTTAGTGCTTTACATGCTGTGCTCTT
TGAAGAGATTTCAACAAGAATATTGTATGTTAAAGCATCAGAGATGGTAATCTAC
AGCTCACCTCTGAAAGCAAATATAAGCTGGGAAAAAAGTTTTGATGAAATTCTTG
AAGTTCATGGTGATCAGTGCAATTGACCTTCTCCCTCACTCCTGCCAGTTGAAAA
TGGATTTTTAAATTATACTGTAGCTGATGAAACTCCTGATTTTGTAGTTAATTTATT
AAGTCTGGGATGTAGAACTTCAAGAAGTAAGAGCTAAGTTCTAAGTTCATGTTTG
TAAATTAATACTTCATTTGGTGCTGGTCTATTTTGATTTTGGGGGGTAATCAGCAT
TATTCTTCAGAAGGGGACCTGTTTTCTTCAAGGGAAGAAACACTCTTATTCCCAA
ACTACAGAATAATGTGTTAAACATGCTAAATAGTTCTATCAGGAAAACAAATCACT
GTATTTATCTCCGCAGGCTATTTGTTCAGAGAGGCCTTTTGTTTAAATATAAATGT
TTAAATATAAATGTTTGTCTGGATTGGCTATAACATGTCTTTCAGCATTAGGCTTT
TAAGAAACACAGGGTTTTGTATTCTTTACTAAAGATATCAGAGCTCTTAATGTTGC
TTAGATGAGGGTGACTGTCAAGTACAAGCAAGACTGGGACCTTAGAAATCATTG
TAGAAACACAGTTTTGAAAGATTTTTACCATGTCTCTAAGCCAACTTTAATTGCTT
AAAAGACATTTTTATTTAGTTGAAAAATCTAGTTTTTTTTGTAAACTGTACCAAATC
TGTATATGTTGTAATAAAACTTATGCTAGTTTATTGGAAGTGTTCAAGAAATAAAA
ATCAACTTGTGTACTGATAAAATACTCTAGCCTGGGCCAGAGAAGATAATGTTCT
TTAATGTTGTCAGGAAACCCTGGCTTGCTTGCCGAGCCTAATGAAAGGGAAAGT
CAGCTTTCAGAGCCAGTGAAGGAGCCACGTGAATGGCCCTAGAACTGTGCCTA
GTTCCTGTGGCCAGGAGGTTGGTGACTGAAACATTCACACAGGGCTCTTGGAT
GGACCCACGAACGCTCTTAGCTTTCTCAGGGGGTCAGCAGAGTTATTGAATCTT
AATTTTTTTTAATGTACAAGTTTTGTATAAATAATAAAGAACTCCTTATTTTGTATT
ACATCTAATGCTTAAGTGTTGCTCTTGGAAAGCTGATGATGTCTCTTGTAGAGAT
GACTCTGAAAAACATTCCAGGAAACCATGGCAGCATGGAGAGCCTCTTAGTGAT
TGTGTCTGCATTGTTATTGTGGAAGATTTACCTTTTCTGTTGTACGTAAAGCTTAA
ATTACTTTTGTTGTGACTTTTTAGCCAGTGACTTTTTCTGAGCTTTTCATGGAAGT
GGCAGTGAAAAATATGTTGAGTGTTCAAAAAAGTGACTGTAATTAATATCTTGCT

GGATTAATGTTTTGTACAATTACTAAATTGTATACATTTTGTTATAGAATACTTTTT
TCTAGTTTCAGTAAATAATGAAAAGGAAGTTAATACCAAC

The target sequence of the MTP gene may be any sequence selected from:

ACTCCCTCACTGGCTGCCATTGAAAGAGTCCACTTCTCAGTGACTCCTAGCTGG
GCACTGGATGCAGTTGAGGATTGCTGGTCAATATGATTCTTCTTGCTGTGCTTTT
TCTCTGCTTCATTTCCTCATATTCAGCTTCTGTTAAAGGTCACACAACTGGTCTCT
CATTAAATAATGACCGGCTGTACAAGCTCACGTACTCCACTGAAGTTCTTCTTGA
TCGGGGCAAAGGAAAACTGCAAGACAGCGTGGGCTACCGCATTTCCTCCAACG
TGGATGTGGCCTTACTATGGAGGAATCCTGATGGTGATGATGACCAGTTGATCC
AAATAACGATGAAGGATGTAAATGTTGAAAATGTGAATCAGCAGAGAGGAGAGA
AGAGCATCTTCAAAGGAAAAAGCCCATCTAAAATAATGGGAAAGGAAAACTTGG
AAGCTCTGCAAAGACCTACGCTCCTTCATCTAATCCATGGAAAGGTCAAAGAGT
TCTACTCATATCAAAATGAGGCAGTGGCCATAGAAAATATCAAGAGAGGTCTGG
CTAGCCTATTTCAGACACAGTTAAGCTCTGGAACCACCAATGAGGTAGATATCTC
TGGAAATTGTAAAGTGACCTACCAGGCTCATCAAGACAAAGTGATCAAAATTAAG
GCCTTGGATTCATGCAAAATAGCGAGGTCTGGATTTACGACCCCAAATCAGGTC
TTGGGTGTCAGTTCAAAAGCTACATCTGTCACCACCTATAAGATAGAAGACAGCT
TTGTTATAGCTGTGCTTGCTGAAGAAACACACAATTTTGGACTGAATTTCCTACA
AACCATTAAGGGGAAAATAGTATCGAAGCAGAAATTAGAGCTGAAGACAACCGA
AGCAGGCCCAAGATTGATGTCTGGAAAGCAGGCTGCAGCCATAATCAAAGCAG
TTGATTCAAAGTACACGGCCATTCCCATTGTGGGGCAGGTCTTCCAGAGCCACT
GTAAAGGATGTCCTTCTCTCTCGGAGCTCTGGCGGTCCACCAGGAAATACCTGC
AGCCTGACAACCTTTCCAAGGCTGAGGCTGTCAGAAACTTCCTGGCCTTCATTC
AGCACCTCAGGACTGCGAAGAAAGAAGAGATCCTTCAAATACTAAAGATGGAAA
ATAAGGAAGTATTACCTCAGCTGGTGGATGCTGTCACCTCTGCTCAGACCTCAG
ACTCATTAGAAGCCATTTTGGACTTTTTGGATTTCAAAAGTGACAGCAGCATTAT
CCTCCAGGAGAGGTTTCTCTATGCCTGTGGATTTGCTTCTCATCCCAATGAAGA
ACTCCTGAGAGCCCTCATTAGTAAGTTCAAAGGTTCTATTGGTAGCAGTGACATC
AGAGAAACTGTTATGATCATCACTGGGACACTTGTCAGAAAGTTGTGTCAGAAT
GAAGGCTGCAAACTCAAAGCAGTAGTGGAAGCTAAGAAGTTAATCCTGGGAGG
ACTTGAAAAAGCAGAGAAAAAAGAGGACACCAGGATGTATCTGCTGGCTTTGAA
GAATGCCCTGCTTCCAGAAGGCATCCCAAGTCTTCTGAAGTATGCAGAAGCAGG

AGAAGGGCCCATCAGCCACCTGGCTACCACTGCTCTCCAGAGATATGATCTCCC
TTTCATAACTGATGAGGTGAAGAAGACCTTAAACAGAATATACCACCAAAACCGT
AAAGTTCATGAAAAGACTGTGCGCACTGCTGCAGCTGCTATCATTTTAAATAACA
ATCCATCCTACATGGACGTCAAGAACATCCTGCTGTCTATTGGGGAGCTTCCCC
AAGAAATGAATAAATACATGCTCGCCATTGTTCAAGACATCCTACGTTTGGAAAT
GCCTGCAAGCAAAATTGTCCGTCGAGTTCTGAAGGAAATGGTCGCTCACAATTA
TGACCGTTTCTCCAGGAGTGGATCTTCTTCTGCCTACACTGGCTACATAGAACG
TAGTCCCCGTTCGGCATCTACTTACAGCCTAGACATTCTCTACTCGGGTTCTGG
CATTCTAAGGAGAAGTAACCTGAACATCTTTCAGTACATTGGGAAGGCTGGTCTT
CACGGTAGCCAGGTGGTTATTGAAGCCCAAGGACTGGAAGCCTTAATCGCAGC
CACCCCTGACGAGGGGGAGGAGAACCTTGACTCCTATGCTGGTATGTCAGCCA
TCCTCTTTGATGTTCAGCTCAGACCTGTCACCTTTTTCAACGGATACAGTGATTT
GATGTCCAAAATGCTGTCAGCATCTGGCGACCCTATCAGTGTGGTGAAAGGACT
TATTCTGCTAATAGATCATTCTCAGGAACTTCAGTTACAATCTGGACTAAAAGCC
AATATAGAGGTCCAGGGTGGTCTAGCTATTGATATTTCAGGTGCAATGGAGTTTA
GCTTGTGGTATCGTGAGTCTAAAACCCGAGTGAAAAATAGGGTGACTGTGGTAA
TAACCACTGACATCACAGTGGACTCCTCTTTTGTGAAAGCTGGCCTGGAAACCA
GTACAGAAACAGAAGCAGGCTTGGAGTTTATCTCCACAGTGCAGTTTTCTCAGT
ACCCATTCTTAGTTTGCATGCAGATGGACAAGGATGAAGCTCCATTCAGGCAAT
TTGAGAAAAAGTACGAAAGGCTGTCCACAGGCAGAGGTTATGTCTCTCAGAAAA
GAAAAGAAAGCGTATTAGCAGGATGTGAATTCCCGCTCCATCAAGAGAACTCAG
AGATGTGCAAAGTGGTGTTTGCCCCTCAGCCGGATAGTACTTCCAGCGGATGGT
TTTGAAACTGACCTGTGATATTTTACTTGAATTTGTCTCCCCGAAAGGGACACAA
TGTGGCATGACTAAGTACTTGCTCTCTGAGAGCACAGCGTTTACATATTTACCTG
TATTTAAGATTTTTGTAAAAAGCTACAAAAAACTGCAGTTTGATCAAATTTGGGTA
TATGCAGTATGCTACCCACAGCGTCATTTTGAATCATCATGTGACGCTTTCAACA
ACGTTCTTAGTTTACTTATACCTCTCTCAAATCTCATTTGGTACAGTCAGAATAGT
TATTCTCTAAGAGGAAACTAGTGTTTGTTAAAAACAAAAATAAAAACAAAACCACA
CAAGGAGAACCCAATTTTGTTTCAACAATTTTTGATCAATGTATATGAAGCTCTTG
ATAGGACTTCCTTAAGCATGACGGGAAAACCAAACACGTTCCCTAATCAGGAAA
AAAAAAAAAAAAAAAAAAGTAAGACACAAACAAACCATTTTTTTCTCTTTTTTTGGA
GTTGGGGGCCCAGGGAGAAGGGACAAGGCTTTTAAAAGACTTGTTAGCCAACT
TCAAGAATTAATATTTATGTCTCTGTTATTGTTAGTTTTAAGCCTTAAGGTAGAAG
GCACATAGAAATAACATC

As used herein, the term "siRNA" means "short interfering RNA", the term "shRNA" refers to "short hairpin RNA". In RNA interference, small interfering RNAs (siRNA) bind the targeted mRNA in a sequence-specific manner, facilitating its degradation and thus preventing translation of the encoded protein. Transfection of cells with siRNAs can be achieved, for example, by using lipophilic agents (among them OligofectamineÔ and Transit-TKOÔ) and also by electroporation.

Methods for the stable expression of small interfering RNA or short hairpin RNA in mammalian, also in human cells are known to the person skilled in the art and are described, for example, by Paul et al. 2002 (Nature Biotechnology 20: 505-508), Brummelkamp et al. 2002 (Science 296: 550-553), Sui et al. 2002 (Proc. Natl. Acad. Sci. U.S.A. 99: 5515-5520), Yu et al. 2002 (Proc. Natl. Acad. Sci. U.S.A. 99: 6047-6052), Lee et al. 2002 (Nature Biotechnology 20: 500-505), Xia et al. 2002 (Nature Biotechnology 20: 1006-1010)..

The siRNA molecules are essentially double-stranded but may comprise 3' or 5' overhangs. They may also comprise sequences that are not identical or essentially identical with the target gene but these sequences must be located outside of the sequence of identity. The sequence of identity or substantial identity is at least 14 and more preferably at least 19 nucleotides long (target sequence). It preferably does not exceed 23 nucleotides. Optionally, the siRNA comprises two regions of identity or substantial identity that are interspersed by a region of non-identity. The term "substantial identity" refers to a region that has one or two mismatches of the sense strand of the siRNA to the targeted mRNA or 10 to 15% over the total length of siRNA to the targeted mRNA mismatches within the region of identity. Said mismatches may be the result of a nucleotide substitution, addition, deletion or duplication etc. dsRNA longer than 23 but no longer than 40 bp may also contain three or four mismatches.

The interference of the siRNA with the targeted mRNA has the effect that transcription/translation is reduced by at least 50%, preferably at least 75%, more preferred at least 90%, still more preferred at least 95%, such as at least 98% and most preferred at least 99%.

A possible system for generating siRNAs would be the one outlined in Miyagishi et al., 2004 (Journal of gene medicine; 6: 715). Figure 22 is adapted from this paper.

The 21 nucleotide-long "stem" is derived from the gene to be silenced and contains 3 or 4 C to U or A to G mutations in the sense strand. Intervening between the inverted repeats is a loop sequence derived from a naturally occuring microRNA. Any unique e.g. 21 base pair sequence from the enzymes of interest could be used to generate these constructs (the sequences of the enzymes are attached). For example:

A useful siRNA for MTP might be:

ATTGTGAAGTGAUUTACCGGGgugugcugucCCUGGUAGGUCCACUUUACAAUUU UU.

A useful siRNA for ACAT might be:

AGCTGGGGTUACCTGGGCCAGgugugcugucCUGGUCCAGGUGACCCCUCUUUU UU

A useful siRNA for DGAT-1 might be:

GGUGGUGGGAGAAGGGGTGCGgugugcugucCGCACCUCUUCUUCCGCCGCCU UUU

A useful siRNA for HMG-CoA reductase might be:

AGTGTTGTUAGGGAAUCCTGGgugugcugucCCAGGGCCUGACAACUUUUUU

Further, the inhibitor can be an antisense nucleic acid molecule specifically hybridizing to a nucleic acid molecule (such as an mRNA) derived from gene encoding a protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell. The term "antisense nucleic acid molecule" refers to a nucleic acid molecule which can be used for controlling gene expression. The underlying technique, antisense technology, can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, J. Neurochem. 56: 560 (1991); "Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression." CRC Press, Boca Raton, FL (1988), or in: Phillips MI (ed.), Antisense Technology, Methods in Enzymology, Vol. 313, Academic Press, San Diego (2000). Triple helix formation is discussed in, for instance, Lee et al., Nucleic Acids Research 6: 3073 (1979); Cooney et al., Science 241: 456 (1988); and Dervan et al., Science 251:

1360 (1991). The methods are based on binding of a target polynucleotide to a complementary DNA or RNA. For example, the 5' coding portion of a polynucleotide that encodes a protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a gene region involved in transcription thereby preventing transcription and the production of the protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell. The term protein refers to proteins and (poly)peptides. The term ((poly)peptide means refers to peptides and polypeptides.

The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into the protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell.

The term "ribozyme" refers to RNA molecules with catalytic activity (see, e.g., Sarver et al, Science 247:1222-1225 (1990)); however, DNA catalysts (deoxyribozymes) are also known. Ribozymes and their potential for the development of new therapeutic tools are discussed, for example, by Steele et al. 2003 (Am. J. Pharmacogenomics 3: 131-144) and by Puerta-Fernandez et al. 2003 (FEMS Microbiology Reviews 27: 75-97). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs for translation of the protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell, the use of trans-acting hairpin or hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, Nature 334:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequence of the mRNA transcribing gene the protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell which will be apparent to the person skilled in the art. Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts. RNase P is another ribozyme approach used for the selective inhibition of

pathogenic RNAs. Ribozymes may be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express the protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of other nucleic acid molecules. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous messages and inhibit translation. Since ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is generally required for efficiency. Ribozyme-mediated RNA repair is another therapeutic option applying ribozyme technologies (Watanabe & Sullenger 2000, Adv. Drug Deliv. Rev. 44: 109-118) and may also be useful for the purpose of the present invention. To this end, catalytic group I introns can be employed in a trans-splicing reaction to replace a defective segment of target mRNA in order to alleviate a mutant phenotype.

In another more preferred embodiment of the present invention, said essential protein of the lipoprotein particle is selected from the group consisting of apolipophorin, Apolipoprotein B100 and Apolipoprotein B48.

In another more preferred embodiment of the present invention the aptamer and the inhibitory antibody are specific for a protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell.

The term "aptamer" refers to RNA and also DNA molecules capable of binding target proteins with high specificity, comparable with the specificity of antibodies. Methods for obtaining or identifying aptamers specific for a desired target are known in the art. Preferably, these methods may be based on the "systematic evolution of ligands by exponential enrichment" (SELEX) process (Ellington and Szostak, Nature, 1990, 346: 818-822; Tuerk and Gold, 1990, Science 249: 505-510; Fitzwater & Polisky, 1996, Methods Enzymol. 267: 275-301). Preferably, said aptamers may be specific for any of the (poly)peptides listed under (1) to (19). The use of aptamers for detection and quantification of polypeptide targets is described in, for example, McCauley et al., 2003, Anal. Biochem., 319:244-250; Jayasena, 1999, Clin.Chem. 45:1628-1650.

The term "antibody" refers to monoclonal antibodies, polyclonal antibodies, chimeric antibodies, single chain antibodies, or a fragment thereof. Preferably the antibody is specific for the protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell. The antibodies may be bi-specific antibodies, humanized antibodies, synthetic antibodies, antibody fragments, such as Fab, $F(ab_2)'$, Fv or scFv fragments etc., or a chemically modified derivative of any of these, all comprised by the term "antibody". Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals with modifications developed by the art. Furthermore, antibodies or fragments thereof specific for the aforementioned proteins can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1998. When derivatives of said antibodies are obtained by the phage display technique, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies Swhich bind to an epitope of the peptide or polypeptide to be analyzed (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). The production of chimeric antibodies is described, for example, in WO89/09622. An inhibitory antibody is an antibody which upon binding interferes with secretion, formation, transport and/or association of lipoproteins with a target cell.

The Figures show:

Figure 1: (A) Schematic picture of CD63:GFP fusion protein. GFP is attached to the C terminus of the protein. (B) CD63:GFP (green) expressed in the wing imaginal disc under the *ap*GAL4 driver, membranes are stained with FM4-64 (red). CD63:GFP is localized to the plasma membrane and large internal vesicles in producing cells, in non-expressing tissue it is found 1 to 3 cell diameters away from producing cells. (C) CD63:GFP (green) colocalizes with lysotracker (red) in enlarged endocytic compartments. (D) CD63:GFP (green) colocalizes with CFPRab5 (blue) in non-expressing tissue, membranes are stained with FM4-64 (red).

Figure 2: (A) schematic picture of *Drosophila* lipophorin. Lipophorin is synthesized as a large precursor, Proapolipophorin, which is then proteolyticaly cleaved into LipophorinI; ApoLI (220kD) and LipophorinII; ApoLII (70kD). (B) Different tissues from 3rd instar larvae were examined for the presence of ApoLII. anti-ApoLII antibody recognizes doublet of expected size in imaginal discs, brain and fat body, in addition a higher molecular weight band is recognized in the fat body that corresponds to unprocessed proapolipophorin. (C) Different tissues from 3rd instar larvae were examined for the presence of ApoLI. anti-ApoLI antibody recognizes a single band of appropriate size in all tissues examined.

Figure 3: Differential centrifugation of homogenates of wild-type larvae or larvae that expressed different GFP fusion proteins. Plasma membrane markers including Thickvein, Cadherin, GFPCD8, GFPCD63 are completely pelleted after 3 hours at 120,000g. Majority of Lipophorin together with soluble cytoplasmic GFP end up in supernatant. Most of gpi-linked proteins (including Fasciclin I (*FasI*), Connectin, Klingon, acetylcholineasterase (*AchE*) and lipid-linked morphogens Wingless and Hedgehog are present in the 120,000g pellet. However, a significant fraction of gpi-linked proteins and lipid-linked morphogens remains in the 120,000g supernatant (S120).

Figure 4: Isopycnic centrifugation in KBr of S120-proteins. (A) SDS-PAGE analysis of fractionated KBr gradient of larval homogenate on 10% Coomassie-blue stained gel. (B) Lipophorin is present in the top, low-density fraction as well as many other gpi-linked proteins, indicating their association with lipidic particles. Upon PI-PLC treatment gpi-linked proteins shift to the high-density fraction, showing that this association is gpi-anchor dependent. (C) Wingless and Hedgehog and their tagged forms are present in low-density fraction of KBr gradient, suggesting that Wingless and Hedgehog associate with lipidic particles. (D) Wingless, Hedgehog and gpi-linked proteins co-immunoprecipitate with lipophorin-based lipidic particles. (E) ApoLII:myc expressed in fat body (*adh*GAL4 driver) and imaginal discs (*ptc*GAL4), respectively. In both cases, ApoLII:myc is present in the top low-density fraction, meaning that it is incorporated into lipidic particles.

Figure 5: Wing imaginal discs are capable of producing lipidic particles. (A) ApoLII:myc expressed in wing imaginal discs is secreted and endocytosed by non-producing cells (*middle*). ApoLI is over-accumulated in the same cells in which ApoLII:myc is over-expressed (*left*). Close-up (*right*) shows ApoLI (red) colocalization with ApoLII:myc (green) in non-expressing tissue. Lipidic particles colocalize with Wingless or Hedgehog. (B,C) ApoLII:myc (green) expressed either in Wingless or Hedgehog-producing cells, strongly colocalizes with Wingless (red) (B, *left*) or Hedgehog (red) (C). Note that CD63:GFP (green) expressed in Wingless producing cells does not colocalize with Wingless (red) (B, *right*)

Figure 6: Lipophorin RNAi. (A) ApoLII levels of the whole larvae extract are reduced by approximately 80% after induction of RNAi comparing to the wild type. (B) Images show overaccumulation of neutral lipids (green) and expansion of lipid droplets (green) in the posterior midgut of lipophorin RNAi larvae (*right*) comparing to the wild type (*left*), membranes are stained red. (C) Lipophorin RNAi discs (*right*) show no dramatic increase in caspase-3 activity comparing to the control (*left*).

Figure 7: Lipophorin depletion reduces the efficiency of Wingless signaling. (A) *Dll* (lower right) levels are strongly reduced in lipophorin RNAi wing imaginal discs (see also lower right in B) comparing to the wild type (upper right). Wingless continues to

be produced (middle). Merged images are shown to left, *dll* in green, *wg* in red. (B) Levels of *hnd* (lower middle) are reduced in sensory organ precursors, some of which are Wingless dependent. Notably, *hnd* continues to be expressed in non-Wingless-dependent sensory organ precursors. Merged images together with *dll* are shown to the left, *hnd* in red, *dll* in green.

Figure 8: Lipid-linked proteins cofractionate with Lipophorin. Western blots of fractionated extracts probed with antibodies to indicated proteins. **A,B)** larval S120's and indicated proportions of larval P120's. AchE=Acetylcholinesterase. **C,D,E)** KBr isopycnic density gradient fractions made from larval (C,D) or disc (E) S120's. Top fraction = 1.14 g/cm^3, bottom fraction = 1.4 g/cm^3. + = PI-PLC-treated, - = mock-treated. **F)** Top panel: P120 and S120 from Hedgehog:HA-expressing discs, probed with anti-HA. Lower panel: P120 and S120 after incubating P120 at 4°C with 5-fold excess of purified lipoprotein particles and recentrifuging. **G,H)** S120's from wild-type or fusion protein-expressing larvae immunoprecipitated with pre-immune, anti-ApoLII, or anti-GFP serum.

Figure 9: Wingless and Hedgehog colocalize with Alexa488Lipophorin (Scale bars = 10 μ). Blue lines = AP boundaries. **A-C)** disc stained with anti-Wingless (A and B red) after 20-minute incubation with Alexa488Lipophorin (B green and C). **D-F)** disc expressing CD63:GFP (B green and F) in Wingless-producing cells stained with anti-Wingless (D and E red). **G-I)** disc stained with anti-Hedgehog (G and H red) after 20-minute incubation with Alexa488Lipophorin (H green and I). **J-L)** disc expressing CD63:GFP (K green and L) in Hedgehog-producing cells stained with anti-Hedgehog (J and K red).

Figure 10: Lipophorin-RNAi perturbs lipid transport. **A)** Extracts from equal numbers of wild-type or *hs-flippase/+; UAS dsRNA/Tubulin:GAL4* larvae probed for ApoLII at indicated hours after heat shock. **B-G)** Posterior midgut (B,C), fat body (D,E) or imaginal discs (F,G) from wild-type (B,D,F) or *hs-flippase/+; Adh:GAL4/+; UAS dsRNA/+* (C,E,G) larvae 5 days after heat shock (AHS). Yellow:Neutral lipid. Red:Plasma membrane. Scale bar = 40μ (B,C) or 10 μ (D-G).

Figure 11: Lipophorin-RNAi alters Hedgehog distribution and signalling. (Blue lines=AP boundary. Scale bar=10 μ.). **A-C)** *dpplacZ/+* disc 4 days AHS stained for LacZ (A,B red) and Collier (B green,C). **D-F)** *hs-flippase/+;dpplacZ/+;Tubulin:GAL4/UAS:dsRNA* disc 4 days AHS stained for LacZ

(D,E red) and Collier (E green,F). **G-I)** wild-type disc 4 days AHS stained for Hedgehog (G,H red) and Patched (H green,I). **(J-L)** *hs-flippase/+;UAS:dsRNA/Tubulin:GAL4* wing disc four days AHS stained for Hedgehog (J,K red) and Patched (K green,L). **M)** Average Collier and DppLacZ staining intensities for 4 wild-type and 4 Lipophorin-RNAi discs. Blue line=AP boundary. Average distance from AP boundary of peak LacZ staining was 16.6 +/- 2.7μ for wild-type, 11.1 +/- 1.5μ for Lipophorin-RNAi.

Figure 12: Hedgehog signalling is unaffected by lipid-depletion. **A,B)** discs from fully fed (A) or lipid-starved (B) larvae. **C,D)** Wings from fully fed (C) or lipid-starved (D) flies. Scale bar = 250μ. **E-J)** discs from fully fed (E-G) or lipid starved (H-J) larvae stained for Hedgehog (E,H and F,I red) and Patched (F,I green and G,J). Scale bar = 30μ. **K-P)** disc from fully fed (K-M) or lipid starved (N-P) *dpplacZ/+* larvae stained for LacZ (K,N and L,O green) and Collier (L,O red and M,P). Blue lines=AP boundary. Scale bars=10μ.

Figure 13: Lipophorin-RNAi narrows the range of Wingless signaling. **A-D)** Apical (A,B) and basolateral (C,D) sections of wild type (A,C) and *Adh:GAL4/+; UASdsRNA/+* (B,D) wing discs 5 days AHS stained for extracellular Wingless. **E,F)** Distalless protein accumulation in wild type (E) and *hs-flippase/+;UAS dsRNA/ TubulinGAL4* (F) wing discs 5 days AHS. **G)** Average Distalless staining intensity with distance from dorsal-ventral boundary of 5 wild type (pink) and 5 *hs-flippase/+;UAS dsRNA/TubulinGAL4* (blue) wing discs. For plots of individual discs, see Figure 19.

Figure 14: Markers for exosomes and Lipophorin in imaginal discs **A-E)** are confocal micrographs of living imaginal discs. Scale bars = 10 m. **A)** disc expressing CD63:GFP (green) in the dorsal compartment under the control of Apterous:GAL4. Plasma membranes are stained red by FM4-64. **B-D)** disc expressing CD63:GFP (B green and C) in the dorsal compartment. Late endosomes are stained with lysotracker (B red and D). **E)** Released CD63 (green) in receiving tissue is present in early endosomes that are marked by CFP:Rab5 (blue). The plasma membrane has been stained red with FM4-64. **F)** Discs (d) and fat bodies (f) from 4 larvae probed with ApoLI . Blue/yellow arrows indicate Pro-apolipophorin and yellow arrows indicate ApoLI. **G)** Discs (d) and fat bodies (f) from 4 larvae probed with ApoLII. Blue/yellow arrows indicate Pro-apolipophorin and blue arrows indicate ApoLII.

Figure 15: Hedgehog and Fasciclin I in low density gradient fractions co-immunoprecipitate with Lipophorin The top fraction of a KBr gradient derived from larval S120 was desalted and immunoprecipitated with either pre-immune or anti-ApoLII serum, then probed with antibodies to ApoLII, Hedgehog or Fas-1. Hedgehog and Fas-1 co-immunoprecipitate with ApoLII.

Figure 16: ApoLI levels are decreased by Lipophorin-RNAi. Anti-ApoLI Western blots of extracts from larvae containing heat shock flippase, Tubulin GAL4 and UAS dsRNA constructs. Equal numbers of larvae were collected and dissolved in loading buffer at the indicated number of hours after heat shock.

Figure 17: Lipophorin-RNAi does not elevate cell death or prevent Insulin signaling. **A,B)** confocal images of living imaginal discs from either wild type (A) or Lipophorin RNAi (B) larvae that ubiquitously produce a Plekstrin homology domain:GFP fusion protein. **C,D)** confocal images of imaginal discs from either wild type (C) or Lipophorin RNAi (D) larvae stained with an antibody to activated Caspase3.

Figure 18: Hedgehog accumulates with Patched in endosomes in Lipophorin-RNAi larvae. **A-D.)** confocal image of an imaginal disc from a Lipophorin RNAi larva stained with antibodies to Patched (A green and C) and Hedgehog (A blue and D). Endosomes were labeled by red dextran uptake (A red and B).

Figure 19: Individual traces of Distalless staining intensity for 5 wild type and 5 Lipophorin RNAi discs. **Figure 20:** Rescue of Hedgehog accumulation in dissected Lipophorin RNAi discs by *in vitro* Lipophorin addition. All scale bars are 10μm. **A-C)** Projection of confocal sections comprising the apical-most 2μm of a Lipophorin RNAi imaginal disc incubated for 2 hours in Graces medium. The disc was stained for Hedgehog (A and C red) and Patched (B and C blue). **D-F)** Projection of confocal sections comprising the apical-most 2μm of a Lipophorin RNAi imaginal disc incubated for 2 hours with purified Lipophorin particles. The disc is stained for Hedgehog (D and F red) and Patched (E and F blue).Discs were stained in parallel and imaged under identical conditions on the same day. Addition of Purified Lipophorin particles at approximately 1/10 the estimated concentration in hemolymph reduces the accumulation of both Patched and Hedgehog in endosomes in the anterior compartment (D-F) compared to those in mock-treated discs (A-C). We sometimes observe that apical, but not basolateral, Hedgehog staining in producing cells is reduced by this treatment – especially in regions more distant from the dv

boundary. **G)** Quantification of Hedgehog puncta in mock-treated and Lipophorin-treated discs. We quantified Hedgehog-positive spots in the anterior compartment within the apical most 5μm of discs using Image J as outlined in Experimental Procedures. The discs were from a single experiment stained in parallel and imaged under identical conditions. The average number of Hedgehog positive spots, standard deviation, and counts in individual discs are shown. This quantification probably underestimates the degree of Lipophorin rescue, because Hedgehog spots in rescued discs are less intense (as well as less numerous) than in mock-treated discs. **H)** Quantification of Patched staining intensity in mock-treated and Lipophorin-treated discs. Projected images of the apical-most 5 μm of each disc were quantified using ImageJ as outlined in materials and methods. 3 mock-treated discs and 5 Lipophorin-treated discs from the same experiment stained in parallel and imaged under identical conditions were averaged to generate the plots shown in the figure.

Figure 21: Wing imaginal discs from wild type or lipophorin RNAi larvae were stained with antibodies to Hedgehog (red0 and Patched(green, lower panel), and with an antibody that detects only the full length unprocessedform of cubutus Interruptus (Ci) (green in lower panel). Inhibiting Lipophorin production prevents cleavage of Ci throughout the anterior compartment. This uncleaved Ci does not activate Patched.

Figure 22: Schematic representation of a proposed siRNA-expression system (adapted from Figure 5 of Miyagishi et al., 2004 Journal of gene medicine; 6: 715).

The following examples illustrate the invention but should not be construed as being limiting.

Example 1: A fraction of gpi-linked proteins, Wingless and Hedgehog does not associate with membranes

To ask in what form Wingless, Hedgehog, and gpi-linked proteins were released from imaginal disc cells, we wished to compare their fractionation with that of exosomes, lipidic particles and free, soluble proteins. To generate a marker for exosomes, we constructed flies expressing a CD63:GFP fusion protein under the control of the GAL4:UAS system (Figure 8A). In vertebrate cells, CD63 localizes predominantly to the internal vesicles of multivesicular endosomes and less strongly to the plasma membrane. Several studies suggest that it is released on exosomes by many cell types. To ask whether CD63:GFP behaves similarly when expressed in *Drosophila* imaginal disc cells, we examined its subcellular localization in this tissue. In producing cells, it is found on the plasma membrane and in large internal structures (Figure 8B). To determine whether these were late endsomes, we stained CD63:GFP-expressing discs with lysotracker, a vital dye that is retained within acidic compartments. CD63:GFP colocalizes extensively with lysotracker in these discs, and even appears to enlarge the endocytic compartment marked by lysotracker (Figure 8C). These data suggest that CD63:GFP is targeted to late endosomes in *Drosophila* cells. To ask whether CD63:GFP-labelled exosomes might be released by imaginal disc cells, we examined non-expressing tissue for GFP fluorescence. We detected CD63:GFP in non-expressing tissue from 1 to 3 cell diameters away from producing cells (Figure 8B). To ask whether released CD63:GFP was endocytosed by neighboring cells, we produced the fusion protein in flies whose early endosomes were labeled by ubiquitously expressed CFP:Rab5. Colocalization between CFP:Rab5 and CD63:GFP in cells that did not express CD63:GFP (Figure 8D) indicates that imaginal disc cells release exosomes that can be endocytosed by their neighbors, and that CD63 is a marker for these exosomes.

As a marker for lipidic particles, we made antibodies to *Drosophila* Lipophorin. Lipophorin is a Lipoprotein containing two protein subunits, LipophorinI and LipophorinII that are generated by cleavage of a larger precursor, Apolipophorin (Figure 2A). Since Apolipophorin is produced mainly in the fat body, we wished to

determine whether Lipophorin particles accumulated in imaginal discs at all. To address this question, we probed Western blots containing different tissues from equal numbers of larvae with antibodies to Lipophorins I and II. Anti- Lipophorin I recognizes a single band of the appropriate size on Western blots from a variety of larval tissues, including imaginal discs (arrow in Figure 2C). Anti-LipophorinII recognizes a doublet of the expected size in discs and brain (arrow in Figure 2B). An additional higher molecular species is present in extracts from fat body, and its size corresponds to that of the unprocessed Proapolipophorin. The presence of Proapolipophorin only in fat body is consistent with its being the primary source of Lipophorin production. Nevertheless, almost equivalent levels of mature Lipophorin are found in many other tissues including imaginal discs, brain and gut.

To examine the fractionation of Wingless, Hedgehog and gpi-linked proteins, we homogenized either wild-type larvae, or larvae that expressed different GFP fusion proteins. We then analyzed these homogenates by differential centrifugation. These experiments showed that plasma membrane and exosomal markers were completely pelleted after centrifugation for 3 hours at 120,000g, whereas the majority of Lipophorin remained in the supernatant (Figure 3). We found that most Wingless:GFP, Hedgehog and gpi-linked proteins (including FasciclinI, GFP:Dally, Connexin, Klingon and Acetylcholineasterase) were present in the 120,000g pellet; this was not unexpected, because these proteins can be found on the plasma membrane or in membrane compartments like endosomes. We were surprised to find, however, that a significant fraction of Wingless:GFP, Hedgehog and gpi-linked proteins remained in the S120 (Figure 3B). These data are consistent with the idea that Wingless:GFP, Hh and gpi-linked proteins in this fraction exist either as free soluble proteins or on lipidic particles.

Example 2: Gpi-linked proteins, Wingless and Hedgehog are present on lipidic particles

To distinguish free proteins from lipidic particles, we subjected proteins in the S120 to isopycnic density centrifugation. Figure 4A shows the Coomasie-stained proteins present in the fractions from such a gradient. Western blotting shows that, under these conditions, Lipophorin moves to low density fractions at the top of the gradient whereas soluble proteins like secreted GFP are present in higher density fractions

(compare the first two panels of Figure 4B). Gpi-linked proteins are found almost entirely in the low-density fraction with Lipophorin, indicating that they are present on a low-density particle (Figure 4B). To ask whether the association of gpi-linked proteins with low density particles depended on the presence of a gpi anchor, we examined the effect of PI-PLC treatment. Treatment with this enzyme prior to density centrifugation causes previously gpi-linked proteins to shift to higher density fractions that contain soluble proteins (Figure 4B). These data suggest that gpi-linked proteins associate with low-density particles via their gpi anchor.

We used the same approach to ask whether the Wingless and Hedgehog proteins might also associate with low-density particles. Figure 4C shows that virtually all the Wingless and Hedgehog present in the S120 co-migrates with Lipophorin in the top fraction of density gradients. This suggests that a fraction of Wingless and Hedgehog is associated with low-density particles.

To determine whether the low-density particle inhabited by these proteins contained Lipophorin, we asked whether Wingless, Hedgehog or gpi-linked proteins could be immunoprecipitated with Lipophorin from the S120. Figure 4D shows that all of these proteins are present in the Lipophorin immunoprecipitate, suggesting that they associate directly or indirectly with Lipophorin. Taken together, these data suggest that the non-membrane-associated fraction of Wingless, Hedgehog and gpi-linked proteins is present on Lipophorin-based lipidic particles.

How might morphogens expressed in imaginal discs come to be associated with Lipophorin? One possibility is that Lipophorin is actually transcribed and translated by imaginal disc cells and associates with Wingless or Hedgehog in the biosynthetic pathway. To ask which larval tissues transcribed *lipophorin*, we performed *in situ* hybridization on whole larvae. Despite the high levels of processed Lipophorin protein found in imaginal discs, brain and gut (in addition to the fat body), *lipophorin* RNA was detected only in cells of the fat body (data not shown), consistent with previous reports. Furthermore, the uncleaved precursor, Apolipophorin, is detected only in the fat body (Figure 2B). This indicates that the Lipophorin synthesized in the fat body is taken up and accumulates in the brain, gut and imaginal discs.

Unlike vertebrate LDL, which is degraded upon uptake, Lipophorin can transfer its lipid cargo without being degraded and is often recycled by insect cells. To investigate whether imaginal discs could construct lipidic particles from exogenously

derived Lipophorin protein, we expressed a myc-tagged version of the 70kd Lipophorin II in either the fat body or imaginal discs using the GAL4 UAS system. Tissues from myc:LipophorinII-expressing larvae were homogenized, and the S120 was subjected to isopycnic density centrifugation. When ApoLII:myc was expressed in the fat body, the majority of the protein was incorporated into low-density particles (Figure 4E). This shows that the tagged construct is functional, and that lipid incorporation is needed to proceed in the context of precursor cleavage. When ApoLII:myc was expressed in imaginal discs, approximately half the protein was incorporated into low-density particles (Figure 4E) Thus, imaginal disc cells, which do not synthesize Lipophorin protein *de novo*, nevertheless possess the machinery to assemble the protein into lipidic particles. Consistent with this, imaginal disc cells do express mRNA encoding Microsomal Triglyceride Transfer Protein, an enzyme required for lipid modification of apolipoprotein B (data not shown). Taken together, these data suggest that fat body-derived Lipophorin that is internalized by imaginal disc cells might be reassembled into new lipidic particles and resecreted.

Lipophorin particles normally contain both protein subunits: Lipophorin I and Lipophorin II. Since ApoLII:myc appeared to be incorporated into low density particles by imaginal disc cells, we wondered whether these particles might also contain endogenous Lipophorin I. To address this question, we examined the distribution of Lipophorins I and II in imaginal discs expressing ApoLII:myc under the control of *patched*GAL4. The over-produced myc fusion protein is detected in *patched*-expressing cells. It is also found in punctate structures in cells outside the expressing region, indicating that it is secreted and possibly endocytosed by surrounding tissue (Figure 5A). Strikingly, cells that over-expressed ApoLII:myc also over-accumulated the fat body-derived Lipophorin I. Furthermore, Lipophorin I was also found in large punctate structures in surrounding tissue that colocalized extensively with ApoLII:myc. Taken together, these data indicate that expressing high levels of ApoLII:myc in disc cells recruits internalized Lipophorin I. They further suggest that both proteins are incorporated into new lipidic particles that are resecreted.

Our data indicate that ApoLII:myc specifically labels those lipidic particles constructed and released by imaginal disc cells. If Wingless and Hedgehog are secreted on lipidic particles, they might be expected to colocalize with myc-tagged Lipophorin produced in the same cells. To address this question, we expressed

ApoLII:myc in either Wingless or Hedgehog-producing tissue and examined the localization of myc, Wingless and Hedgehog. We found very strong (on average 67%) colocalization between myc and Wingless and between myc and Hedgehog in punctate structures outside the expressing region. These data are consistent with the release of at least a fraction of Wingless and Hedgehog on Lipophorin particles (Figure 5B and C).

To determine whether association of Wingless with Lipophorin was required for Wingless signaling activity, we wished to examine the consequences of Lipophorin depletion using RNA interference. In order to allow both temporal and spatial control of dsRNA expression, we utilized a modified form of the GAL4:UAS system in which the *lipophorin*-derived inverted repeat was separated from the promoter by a removable cassette containing the HcRed gene. Upon heat shock-mediated excision of the HcRed cassette, *lipophorin* dsRNA was expressed at specific times in specific tissues. Production of *lipophorin* dsRNA either ubiquitously or specifically in the fat body was lethal, even when expression was not initiated until the third larval instar. Lipophorin protein levels in these animals were reduced by approximately 80% five days after heat shock (Figure 6A). Consistent with the observation that *lipophorin* is not expressed in imaginal discs, no phenotype was ever observed in adult flies when *lipophorin* dsRNA was expressed in this tissue (data not shown).

Lipophorins in other insects transport lipid from the gut to the fat body and from the fat body to other tissues. To determine the effects of Lipophorin reduction on lipid transport, we assessed the levels of neutral lipids in different tissues by staining them with Nile Red. The posterior midgut is thought to mediate nutrient uptake, and droplets containing neutral lipids are apparent specifically within cells of this region (Figure 6B). Lipophorin reduction causes overaccumulation of neutral lipid in and a dramatic expansion of these droplets (Figure 6B). Thus, decreased levels of Lipophorin slow the rate of lipid transport from the midgut. The fat bodies of larvae with reduced Lipophorin levels are smaller in size, but still contain neutral lipid-rich droplets. This suggests that the transport of lipid from the gut to the fat body is reduced but not eliminated in these animals, or the reduction in lipid export from the fat body balances the decreased lipid import. Imaginal discs normally contain abundant lipid droplets that stain with Nile Red. Reduction of Lipophorin levels reduces, but does not eliminate, the lipid in these organelles. Thus, the partial

Lipophorin depletion caused by RNA interference reduces but does not eliminate lipid traffic between gut, fat body and imaginal discs.

Because *lipophorin* is not expressed in the discs, it is not possible to restrict the depletion of Lipophorin to this tissue. Thus we are only able to study the requirement for Lipophorin in morphogen signaling in the context of systemic reduction of Lipophorin levels. Wing imaginal discs from Lipophorin RNAi larvae are somewhat smaller than normal, especially in the wing pouch. To ask whether their smaller size was the result of increased apoptosis, we compared the level of activated caspase with that of wild type discs (Figure 6C). Discs from Lipophorin RNAi larvae showed no increase in caspase activity, suggesting that cells had not been eliminated by apoptosis.

To determine the efficiency of Wingless signaling in discs with reduced Lipophorin, we stained them for Wingless, Distalless and Hindsight. Distalless is produced in a broad region on either side of the dorsal-ventral compartment boundary in response to Wingless signaling (Figure 7A and B). Hindsight is additionally expressed at a high level in sensory organ precursors some of which are Wingless-dependent (Figure 5B). Although Wingless continues to be produced (Figure 5A) both Distalless and Hindsight are strongly reduced in discs from larvae expressing Lipophorin dsRNA (Figure 5A and B). Significantly, Hindsight continues to be expressed in the non-Wingless-dependent sensory organ precursors. This shows that the lower levels of neutral lipid in these discs is not sufficient to perturb Hindsight expression, and suggests that Lipophorin depletion reduces the efficiency of Wingless signaling.

Example 3: Lipid-linked proteins copurify with Lipophorin

We compared sedimentation of Wingless, Hedgehog and gpi-linked proteins to that of transmembrane proteins, exosomes, and Lipophorin particles. To mark exosomes, we used flies expressing a vertebrate CD63:GFP fusion construct. CD63 is a tetraspanin that localizes to internal vesicles of multivesicular endosomes, and is released on exosomes 17,18. In Drosophila imaginal discs, CD63:GFP localizes to late endosomes in producing cells, consistent with vertebrate studies (Figure 14A-D). It is released and endocytosed by neighboring cells between 1 and 3 cell diameters away (Figure 14A,E), indicating that it is present on exosomes.

To mark lipoprotein particles, we made antibodies to Drosophila Apolipophorins I and II (ApoLI and ApoLII); these proteins are generated by cleavage of the precursor pro-Apolipophorin 19,20. Lipophorin is produced in the fat body 20; consistent with this, we can not detect apolipophorin transcripts in imaginal discs (data not shown). Nevertheless, the ApoLI and ApoLII proteins are as abundant in discs as in the fat body (Figure 14F and G).

Plasma membrane and exosomal markers are completely pelleted after centrifugation for 3 hours at 120,000g, whereas most ApoLII remains in the supernatant (Figure 8A). Most Wingless:GFP and Hedgehog is present in the pellet, as are the gpi-linked proteins Fasciclin I 21, Connectin 22, Klingon 23 and Acetylcholineasterase 24 (Figure 8B); this is not unexpected, because these proteins localize to the plasma membrane and internal membrane compartments. Surprisingly, however, some Wingless:GFP (6%), Hedgehog (2%) and gpi-linked proteins (14-22%) remain in the supernatant (Figure 8B).

The S120 contains both free soluble proteins and lipoprotein particles. To separate them, we performed isopycnic density centrifugation. In these gradients, Lipophorin moves to the top low-density fraction whereas soluble proteins are present in higher density fractions (first two panels of Figure 8C). Gpi-linked proteins are found almost entirely in the top fraction with Lipophorin. Treating the S120 with Phosphatidylinositol-specific Phospholipase C (PI-PLC) prior to density centrifugation shifts their migration to higher density fractions (Figure 8C). This suggests that gpi-linked proteins associate with low-density particles via their gpi-anchor.

Similarly, when S120's from larvae that express Wingless:GFP or Hedgehog:HA in imaginal discs are subjected to isopycnic density centrifugation, these proteins are found in the lowest density fraction with ApoLII, as is endogenous Hedgehog (Figure 8D). Antibodies to endogenous Wingless detect a doublet in the top fraction and a band of somewhat higher mobility in high-density fractions. These data indicate that non-membrane-bound Wingless and Hedgehog associate with low-density particles in imaginal discs in vivo; other larval tissues may secrete Wingless in a non-Lipophorin-associated form.

We worried that Lipophorin particles in the hemolymph might extract proteins from discs during larval homogenization, so we repeated these experiments using dissected discs. All Wingless, Hedgehog and ApoLII in the imaginal disc S120's are

present on low-density particles (Figure 8E), suggesting that their association is not an artefact of homogenization. Consistent with this, incubating pelleted imaginal disc membranes with an excess of purified Lipoprotein particles does not extract Hedgehog:HA from membranes under the conditions used for homogenization (Figure 8F). This suggests association of lipid-linked morphogens with Lipophorin depends on active cellular processes and does not occur during extract preparation.

To ask whether lipid-linked proteins associated with Lipophorin, or with some other low-density particle, we immunoprecipitated ApoLII from larval S120's and probed precipitates for Wingless, Hedgehog or GFPgpi. These proteins are immunoprecipitated by anti-ApoLII, but not pre-immune serum (Figure 8G). Furthermore, anti-ApoLII is unable to precipitate secreted GFP that does not contain a gpi anchor (Figure 8H). Hedgehog and Fas-1 also immunoprecipitated with ApoLII from the more purified top fraction of KBr gradients (Figure 14). Thus, lipid-linked morphogens and gpi-linked proteins associate directly with Lipophorin particles.

Example 4: Morphogens colocalize with Lipophorin

These experiments do not exclude the possibility that some Wingless or Hedgehog in the P120 might be present on exosomes. To investigate this, we expressed CD63:GFP in either Wingless or Hedgehog producing cells and looked for colocalization with CD63:GFP-labelled exosomes in receiving tissue. No significant colocalization is detected (Figure 9D-F and J-L). Thus, it seems unlikely that imaginal disc cells release Wingless or Hedgehog on exosomes, although the mechanism remains a possibility for transmembrane ligands such as Boss or Notch 25,26.

To test whether Wingless or Hedgehog colocalized with lipoprotein particles, we incubated imaginal discs with purified Lipophorin particles fluorescently labelled with Alexa488; although they work for Western blotting, neither anti-ApoLI nor ApoLII antibodies detect endogenous Lipophorin by immunofluorescence. Immunostaining reveals that Wingless and Hedgehog are found in the same endosomes as Alexa488Lipophorin (Figure 9A-C, G-I). Unsurprisingly, Lipophorin uptake is not limited to areas where these morphogens are abundant; Lipophorin has a nutritional function as well and many potential receptors are encoded in the genome 27. Strong colocalization between Lipophorin and lipid-linked morphogens is predicted if Wingless and Hedgehog are endocytosed with Lipophorin. Nevertheless, we cannot

exclude the possibility that these proteins were internalized separatedly and converged in the same endosomes.

Example 5: Lipophorin-RNAi perturbs lipid transport

To assess the role of Lipophorin in larval growth and development, we reduced the levels of ApoLI and II by RNA-interference directed against two different regions of the apolipophorin mRNA. Similar phenotypes were produced by each construct. To express dsRNA, we used a modified GAL4:UAS system in which expression of inverted repeats can be temporally controlled by heat shock-dependent excision of an intervening HcRed cassette by the FLP recombinase. We tested extracts from wild type larvae or larvae harbouring hs-flp, GAL4 driver and UAS dsRNA constructs at various times after heat shock to see how fast Lipophorin levels were reduced (Figure 10A). Larvae of the latter genotype made only 50% of the wild type level of ApoLII, even in the absence of heat shock; basal activity of the heat shock promoter in the fat body causes HcRed excision in approximately 50% of fat body cells, although excision strictly depends on heat shock in other larval tissues (data not shown). Although they survive less frequently, these flies have no obvious phenotype.

After heat shock, all fat body cells excise the HcRed cassette and ApoLII levels decrease further. After four days, ApoLII is reduced to 5% of wild type levels. ApoLI levels are reduced with similar kinetics (Figure 16). These animals prolong the third larval instar and rarely pupariate. We performed all the experiments described below on third instar larvae 4-6 days after heat shock.

To investigate the requirement for Lipophorin in lipid transport, we assessed the accumulation of neutral lipids in larval tissues by staining them with Nile Red.28 Cells of the posterior midgut normally contain many small lipid droplets (Figure 10B). Lipophorin reduction causes a dramatic expansion of these droplets (Figure 10C), suggesting that Lipophorin is required for the efficient extraction of lipid from the midgut.

Wild type fat body contains both small and large lipid droplets (Figure 10D). Fat bodies of Lipophorin-RNAi larvae are reduced in size and have fewer small lipid droplets (Figure 10E), although larger droplets appeared normal. These data suggest that Lipophorin delivers lipid to the fat body.

Lipid droplets in discs from Lipophorin-RNAi larvae are fewer and smaller than in wild type (compare Figure 10F and G). Their discs are also reduced in size, particularly in the wing pouch (data not shown). Thus, discs require Lipophorin for accumulation of lipid droplets and for growth. Neither Caspase3 activation nor membrane PIP3 accumulation is altered in Lipophorin-RNAi discs (Figure 17), suggesting their small size is not due to cell death or reduced Insulin signalling 29.

Example 6: Hedgehog function requires Lipophorin

To test whether Lipophorin association was required for Hedgehog function, we examined Hedgeghog distribution and signalling in Lipophorin-RNAi larval discs. In wild type discs, Hedgehog expressed in the posterior compartment moves across the anterior-posterior (AP) compartment boundary and activates transcription of short and long-range target genes. Cells closest to the source respond by activating the transcription of collier (Figure 11B,C) and patched (Figure 11H,I). Further away, Hedgehog activates transcription of decapentaplegic (Figure 11A,B) 1,30,31. We monitored levels of Collier and a decapentaplegic reporter construct (dpplacZ) in wild type and Lipophorin RNAi discs stained in parallel and imaged under identical conditions. Discs from Lipophorin RNAi larvae activate collier at least as efficiently as those of wild type (compare Figure 11C and F). In contrast, the range of activation of dppLacZ is significantly narrowed in lipophorin RNAi discs. dppLacZ is expressed up to 11 cells away from the AP boundary in wild type discs (Figure 11A, B), but only up to 6 cells away in Lipophorin RNAi larvae (Figure 11D, E,M). These data suggest that Lipophorin knock-down decreases the range of Hedgehog signalling.

To ask whether Hedgehog trafficking was altered, we stained discs for Hedgehog and Patched. In wild type discs, Hedgehog moves into the anterior compartment where it is found in endosomes, often with Patched 32,34 (Figure 11G-I). Patched-mediated endocytosis is thought to sequester Hedgehog and limit its spread 32,33. Hedgehog is most abundant up to 5 cell rows away from the AP boundary; although Hedgehog signals over a wider range, specific staining there cannot be distinguished from background. In Lipophorin-RNAi discs, Hedgehog (Figure 11J,K) accumulates to abnormally high levels in the first 5 rows of anterior cells. We counted 380 Hedgehog spots in the most apical 10 microns of the wild type disc shown in Figure 11G. The Lipophorin RNAi disc shown in Figure 11J contains 1208 Hedgehog spots

in the same region. Most accumulated Hedgehog colocalizes with Patched (Figure 11K,L) in endosomes (Figure 18). Furthermore, Patched co-accumulates more extensively with Hedgehog in endosomes than it does in wild type (Figure 11 H, K). These data indicate that Lipophorin RNAi either increases the susceptibility of Hedgehog to Patched-mediated endocytosis, or prevents subsequent degradation of the protein.

We wondered whether Lipophorin-depletion might affect Hedgehog trafficking indirectly by preventing release of a needed co-factor from some other larval tissue. To investigate this, we added purified Lipophorin particles to explanted Lipophorin-RNAi discs and examined Hedgehog and Patched distribution. Abnormal Hedgehog and Patched accumulation was strongly reduced by a two-hour incubation of dissected discs with Lipophorin particles (Figure 20). Thus Lipophorin acts directly in imaginal discs to control Hedgehog trafficking, although it is still possible that its effects on signalling are indirect.

Drosophila cannot synthesize sterols and relies on dietary sources. To assess whether reduced uptake of sterols or other lipids might cause the changes we see, we explored the effects of lipid deprivation on larval development. Larvae were allowed to hatch and feed on sucrose/agarose plates supplemented with yeast for 2-3 days, then transferred to plates containing chloroform-extracted yeast autolysate, rather than yeast. These larvae are developmentally delayed; after 7 days of lipid deprivation, their discs are much smaller than those of younger late third instar larvae (compare Figure 12 A, B). In contrast, yeast-fed siblings pupariate and begin to eclose by this time. Those flies that infrequently eclose after larval lipid depletion are small (35-60% of normal body weight) but normally patterned (Figure 12C, D). Thus, lipid depletion stalls imaginal growth.

To ask whether lipid starvation affected Hedgehog trafficking or signalling, we deprived larvae of lipid 2 days after hatching and stained their discs 6 days later (Figure 12H-J). No changes in Hedgehog or Patched distribution are apparent in these discs compared with younger yeast-fed discs of similar size (Figure 12E-G). Furthermore, the range of dpp and collier expression does not differ in lipid starved and yeast-fed discs (Figure 12K-P). Thus, lipid starvation does not mimic the effects of Lipophorin knock-down. We speculate that lipid starvation-induced growth arrest prevents membrane sterol from dropping to levels that would interfere with the

Hedgehog pathway. Thus, Lipophorin does not indirectly affect the Hedgehog pathway via lipid deprivation.

Example 7: Wingless function requires Lipophorin

To ask whether Lipophorin RNAi perturbed Winglesss trafficking, we examined Wingless distribution. In Lipophorin-RNAi discs, extracellular Wingless is less abundant on both the apical and basolateral epithelial surfaces and spreads over shorter distances (Figure 13A-D). However, no consistent alterations in intracellular Wingless are detected (not shown). Thus, Lipophorin promotes accumulation of extracellular Wingless.

To investigate whether Wingless signalling required Lipophorin, we examined the activation of two target genes. Senseless is produced only in cells near the Wingless source and its expression is unaffected by Lipophorin RNAi (not shown). Distalless is normally produced in a gradient throughout most of the wing pouch. In Lipophorin-RNAi discs, the Distalless gradient is abnormally narrow (Figure 13E-G). This suggests that Lipophorin knock-down specifically perturbs long-range Wingless signalling.

Example 8: Methods used in Examples 3 to 7

Fractionation

5 ml larvae were homogenized with 5 ml 150 mM NaCl, 50 mM Tris-Cl pH 7.4, 2 mM EGTA plus protease inhibitors on ice. The 1000g supernatant was centrifuged 3 hours at 33,600 rpm (120,000 g) at 4°C in a SW40Ti rotor generating a pellet (P120) and supernatant (S120). For isopycnic density centrifugation, we added 0.33g/ml KBr to the S120, and centrifuged 2 days at 40,000 rpm (285,000 g) at 10°C in an SW40Ti rotor.

Immunoprecipitation

S120 pre-cleared 2 hours with protein A-sephacryl CL4B beads was incubated with beads linke to different sera. Beads were washed with PBS, 1% BSA, then PBS, and eluted using Laemmli sample buffer or 150 mM NaCl, 2 mM EDTA, 100 mM Tris-Cl pH 8,3, 0.5% Nonidet -P40, 0.5% sodiumdeoxycholate and 0.1% SDS.

Antisera

Rabbits were immunized with synthetic peptide LEGVIRRDSPKFKDL (Hedgehog amino acids 123-138), conjugated to keyhole limpet hemocyanin (Eurogentec, Seraing, Belgium). Antibody was affinity-purified on peptide-conjugated affigel-15 columns (Biorad, Hercules, CA).

DNA encoding amino acids 195-509 or 891-1070 (parts of ApoLII and ApoLI, respectively) was amplified from GH18004 (Resgen) and cloned into pQE30. His-tagged fusion proteins (Qiagen, Valencia, CA) were used to immunize rats or rabbits.

Expression construct

CD63:EGFP amplified from pEGFP-C1-bos (gift from Gillian Griffiths) was cloned into pUAST [45].

RNA interference

RNA-interference was induced by expressing inverted repeats derived from two different regions of the Pro-apolipophorin cDNA (607 bp ending 47 bp from stop codon, and 500 bp starting at ATG). The first was amplified and inserted into pENTR2B (Invitrogen, Leek, The Netherlands). Using the Gateway system, we inserted it twice in inverted orientation into pFRIPE. pFRIPE is derived from pUAST; downstream of the UAS are two Gateway insertion sites flanking an FLP cassette containing the HcRed gene and a transcription termination sequence.

The second fragment was amplified and cloned as an inverted repeat into pUhr. pUhr was derived from pUAST by inserting an HcRed-containing FLP cassette between the UAS and the multiple cloning site.

Flies containing Lipophorin RNAi constructs were crossed with others harbouring heat shock-inducible Flippase and one of several GAL4 drivers. After 5 days at 25°C, larvae were heat shocked for 90 minutes at 37°C; this causes excision in all cells as determined by HcRed fluorescence. No excision occurs without heat shock in any larval tissue except the fat body (see results).

dsRNA expressed under the control of either TubulinGAL4 (ubiquitous), AdhGAL4 (fat body and part of the gut) or C765GAL4 (disc-specific) was semi-lethal and produced identical larval phenotypes. No phenotype was ever observed when *lipophorin* dsRNA was expressed in imaginal discs.

Immunohistochemistry

Imaginal discs were fixed and stained as described [13]. Antibodies were diluted as follows: anti-Wg [46], 1:200; anti-Hh [47], 1:500; 1:100; anti-Ptc [48] 1:50; anti-βgal (Promega Z378A) 1:100; anti-Col [31]1:200. To compare wild type and Lipophorin RNAi animals, tissues were stained in parallel and imaged under identical conditions with an LSM Zeiss or Leica confocal microscope.

Image Analysis

Hedgehog-positive spots in wild type and Lipophorin-RNAi discs were quantified in 10 confocal sections 1μ apart. Signal threshold was adjusted to 130 and images were despeckled using ImageJ. Grids were overlaid on the processed image and spots were counted manually.

We used ImageJ to quantify the Hedgehog signalling range in 5 projected apical sections of Col and Dpp stained discs. For each image, we determined pixel intensity along 10 lines centered at the AP boundary using Plot Profile and averaged them to obtain a plot for each disc. Average plots from 4 discs of each type were generated using Microsoft Excel. Distalless range in Figure 13 and Patched staining in Figure 20 were quantified similarly.

Lipid Starvation

Eggs were collected on apple juice/agar plates + yeast for 24 hours, then allowed to develop for 2-3 days on the same yeast-containing plates. Larvae were rinsed with PBS + 0.05% TritonX100, treated for 10 seconds with 50% Na hypocholorite, and rinsed with sterile H_20. Larvae were transferred with sterile forceps to 10 cm plates containing 2% chloroform extracted agarose, 2.5% sucrose and 0.15% Nipagen, supplemented with either 0.3g chloroform extracted yeast autolysate (for lipid starvation), or 0.3g yeast (for lipid-fed controls).

Labelling Lipophorin with Alexa488

Lipophorin particles were fluorescently labeled with Alexa Fluor 488 (Molecular Probes) according to manufacturer's instructions. Conjugate was separated from un-reacted label using Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) and eluted with 100 mM Na-Phosphate, pH7.4, 100 mM NaCl, 10% sucrose.

Incubation of dissected discs with Lipophorin particles

For experiments shown in Figure 9 and Figure 14, imaginal discs were incubated at 29°C with 50 µg/ml Lipophorin particles for 20 minutes and 2 hrs, respectively. Based on the starting volume of larvae and the final volume in which Lipophorin was eluted, we estimate that this represents approximately 1/10 of the concentration of Lipoprotein particles present in the hemolymph.

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Claims

1. Use of an inhibitor of Microsomal Triglyceride Transfer Protein (MTP), HMG-CoA reductase, DGAT and/or ACAT for the preparation of a pharmaceutical composition for the treatment of tumors.

2. Use of an inhibitor of lipoprotein secretion for the preparation of a pharmaceutical composition for the treatment of tumors.

3. Use of an inhibitor of lipoprotein for the preparation of a pharmaceutical composition for the treatment of tumors.

4. Use of an inhibitor of lipoprotein transport for the preparation of a pharmaceutical composition for the treatment of tumors.

5. Use of an inhibitor of lipoprotein association with a target cell for the preparation of a pharmaceutical composition for the treatment of tumors.

6. Use of an inhibitor of the association of a protein of the Wnt or Hedgehog family with lipoproteins for the preparation of a pharmaceutical composition for the treatment of tumors.

7. The use of any one of claims 2 to 6, wherein said lipoprotein is very low density lipoprotein (VLDL) or low density lipoprotein (LDL).

8. The use of any one of claims 1 to 7, wherein said tumor is a malignant tumor.

9. The use of any one of claims 1 to 8, wherein growth and/or progression of said tumor are caused by one or more protein of the Wnt or Hedgehog family.

10. The use of any one of claims 1 to 9, wherein said tumor over-expresses one or more protein of the Wnt or Hedgehog family.

11. The use of any one of claims 6 to 10, wherein said protein of the Wnt family is Wnt1, Wnt2, Wnt2B, Wnt3, Wnt3A, Wnt4, Wnt5A, Wnt5B, Wnt6, Wnt7A, Wnt7B, Wnt8A, Wnt8B, Wn9A, Wnt9B, Wnt10A, Wnt10B, Wnt11 and/or Wnt16 and/or said protein of the Hedgehog family is Desert Hedgehog, Indian Hedgehog and/or Sonic Hedgehog.

12. The use of any one of claims 1 to 9 or 11, wherein said tumor is selected from the group consisting of esophageal tumor, biliary tract tumor, gastric tumor, pancreatic tumor and malignant melanoma.

13. The use of any one of claims 1 to 11, wherein said tumor is selected from the group consisting of gastric tumor, colorectal tumor, pancreatic tumor, esophageal tumor, squamous cell carcinoma, cervical tumor and malignant melanoma.

14. The use of any one of claims 1 or 7 to 13, wherein said inhibitor of MTP is selected from the group consisting of diaminoindanes such as 8aR or 19aR; citrus flavonoids such as naringenin or hesperetin; quercetin; BMS-197636; BMS-201038; BMS-200150; other benzimidazole-based MTP inhibitors; CP-346086 and Implitapide (BAY-13-9952).

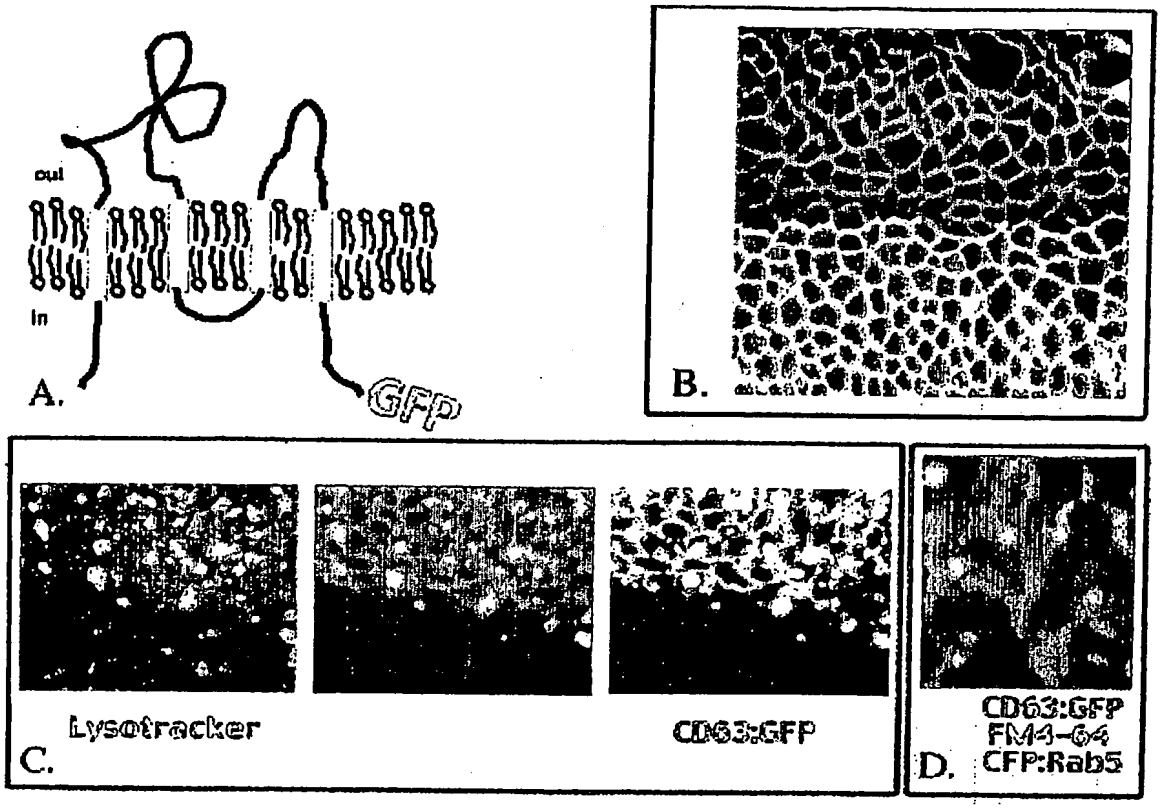
15. The use of any one of claims 1 or 7 to 13, wherein said inhibitor is selected from the group consisting of CI-1-11, FCE 27677, DuP 128, CI-976, PD-138142-15, CP-113818, HL-004, FR-145237, TMP-153 (chemical formula = N-[4-(2-chlorophenyl)-6,7-dimethyl-3-quinolyl]-N'-(2,4-difluorophe nyl)urea, atorvastatin (lipitor), simvastatin, lovastatin, cerivastatin, pravastatin, fluvastatin, mevastatin (compactin), rosuvastatin, pitavastatin and Gemfibrozil 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid.

16. The use of any one of claims 1 to 13, wherein the inhibitor is selected from siRNA, shRNA, ribozyme, antisense nucleic acid molecule, aptamer or inhibitory antibody.

17. The use of claim 16, wherein the siRNA, shRNA, ribozyme and antisense nucleic acid molecule have a region of complementarity with at least one gene encoding a protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell.

18. The use of claim 17, wherein said essential protein is selected from the group consisting of apolipophorin, Apolipoprotein B100 and Apolipoprotein B48.

19. The use of claim 16, wherein the aptamer and the inhibitory antibody are specific for a protein essential for lipoprotein secretion, formation, transport and/or association of lipoproteins with a target cell.



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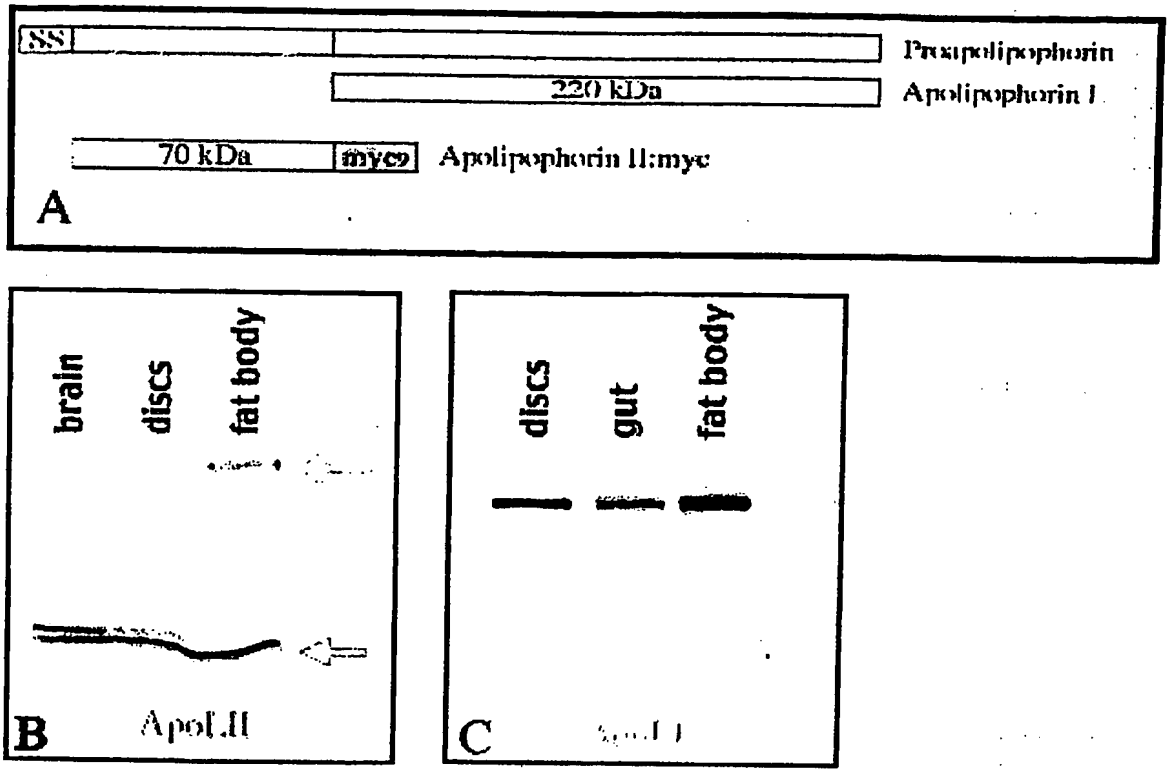
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Apolipophorin I
70 kDa
myc9
Apolipophorin II:myc
A
brain
discs
fat body
B
ApoLII
discs
gut
fat body
C

Figure 2

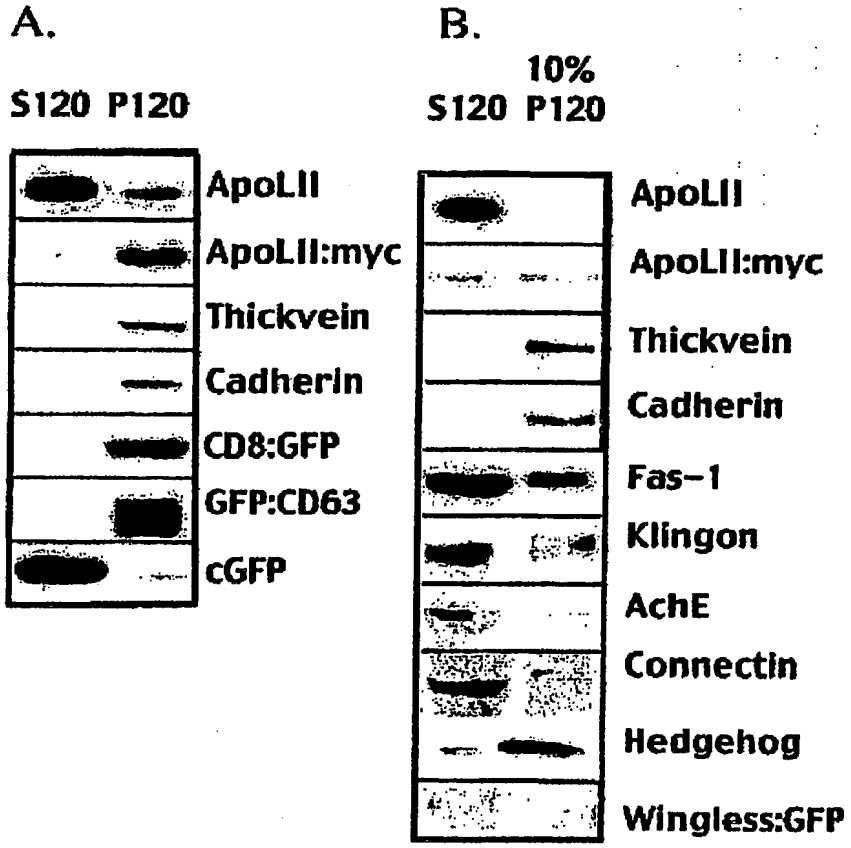

A.
S120 P120
ApoLII
ApoLII:myc
Thickvein
Cadherin
CD8:GFP
GFP:CD63
cGFP
B.
10%
S120 P120
ApoLII
ApoLII:myc
Thickvein
Cadherin
Fas-1
Klingon
AchE
Connectin
Hedgehog
Wingless:GFP

Figure 3

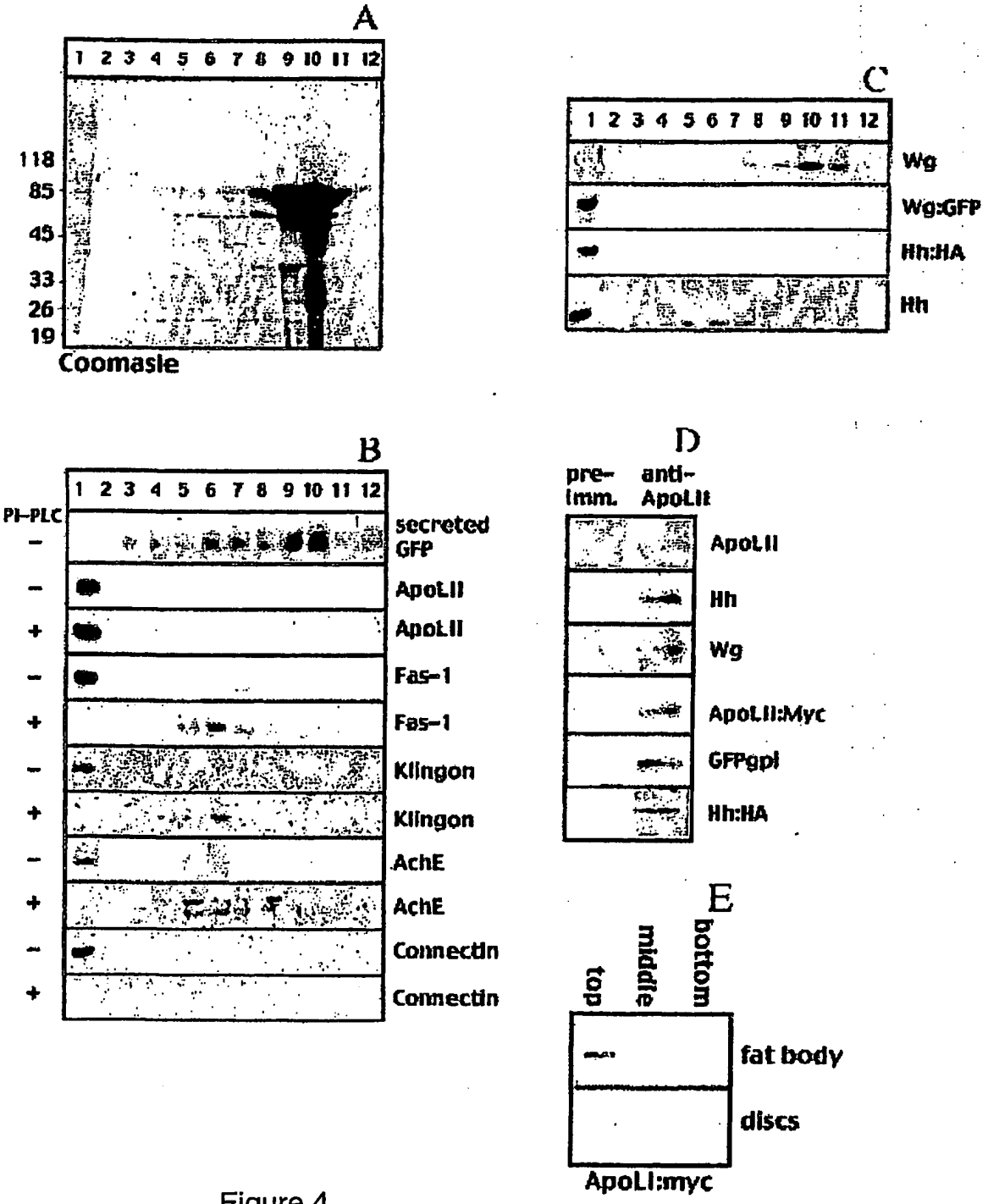

A
1 2 3 4 5 6 7 8 9 10 11 12
118
85
45
33
26
19
Coomasie
B
1 2 3 4 5 6 7 8 9 10 11 12
PI-PLC
secreted GFP
ApoLII
ApoLII
Fas-1
Fas-1
Klingon
Klingon
AchE
AchE
Connectin
Connectin
C
1 2 3 4 5 6 7 8 9 10 11 12
Wg
Wg:GFP
Hh:HA
Hh
D
pre-imm.
anti-ApoLII
ApoLII
Hh
Wg
ApoLII:Myc
GFPgpi
Hh:HA
E
top
middle
bottom
fat body
discs
ApoLI:myc

Figure 4

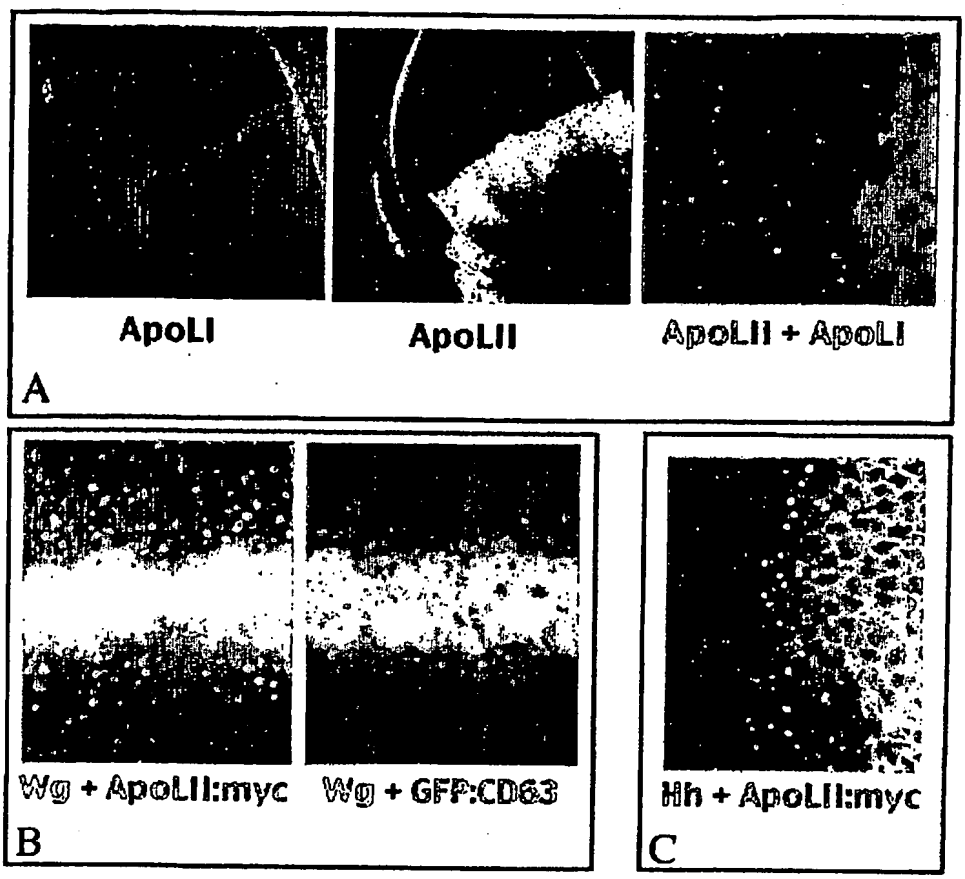
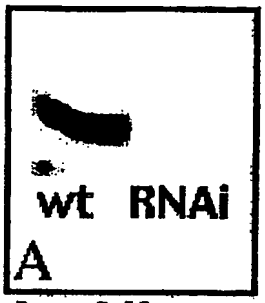
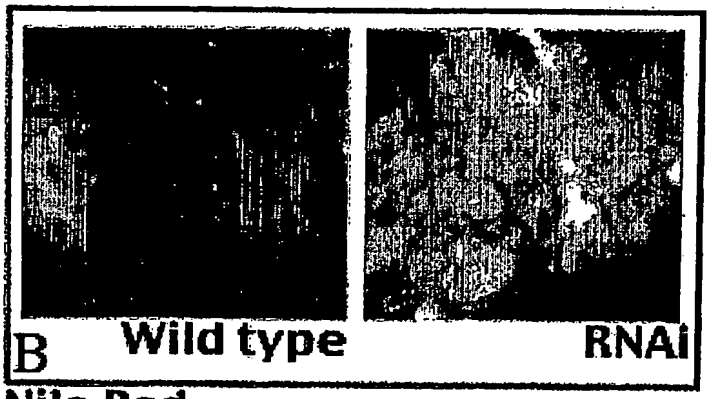

ApoLI
ApoLII
ApoLII + ApoLI
A
Wg + ApoLII:myc
Wg + GFP:CD63
B
Hh + ApoLII:myc
C

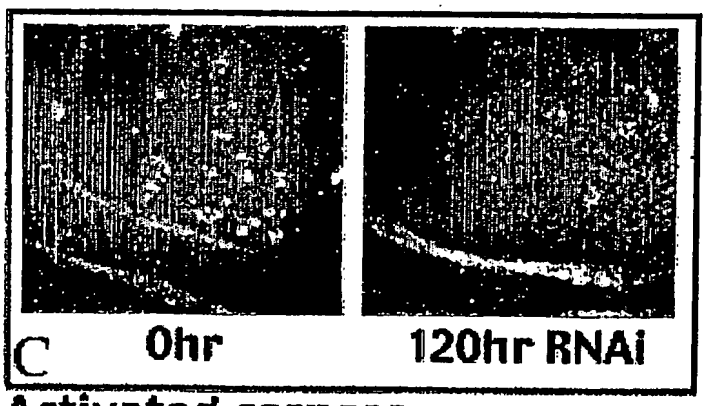
Figure 5



ApoLII

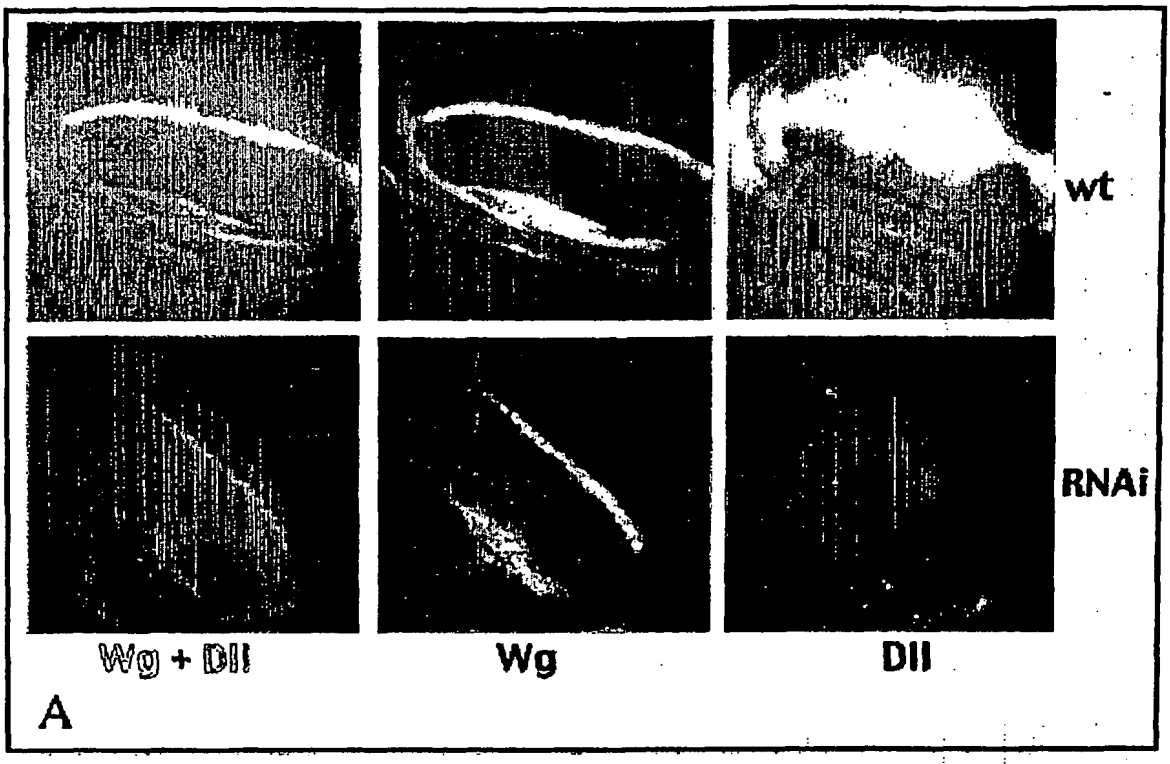


Nile Red



Activated caspase

Figure 6



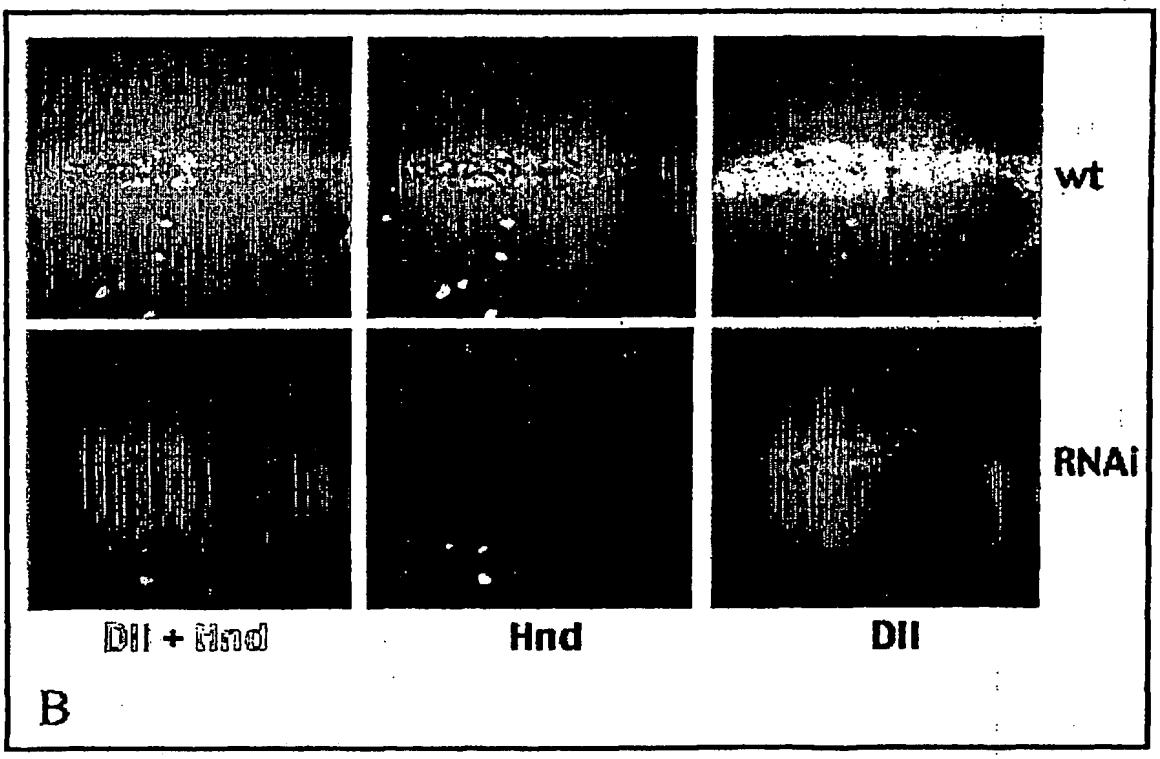


Figure 7

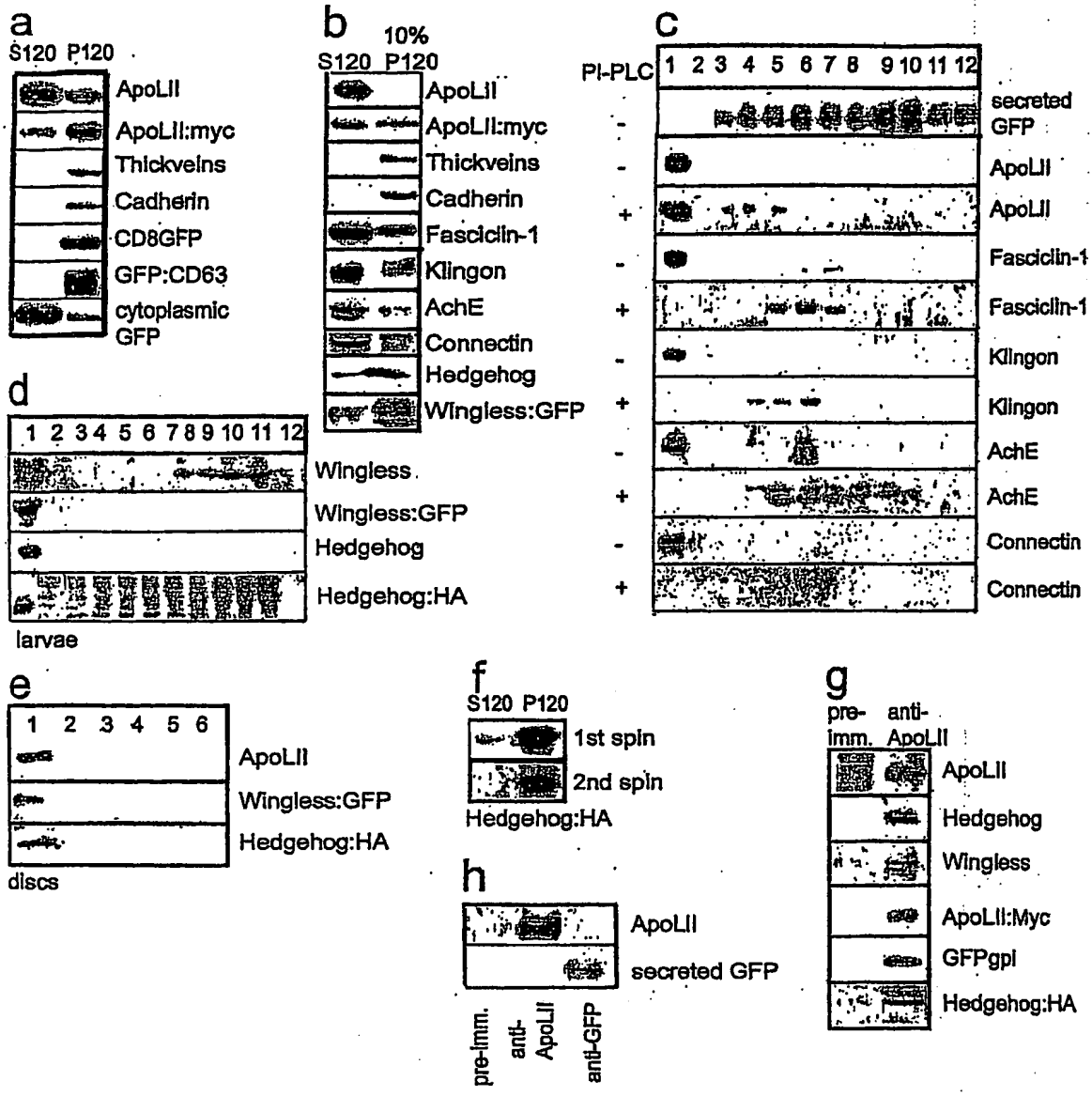

a
S120 P120
ApoLII
ApoLII:myc
Thickveins
Cadherin
CD8GFP
GFP:CD63
cytoplasmic GFP
b
10%
S120 P120
ApoLII
ApoLII:myc
Thickveins
Cadherin
Fasciclin-1
Klingon
AchE
Connectin
Hedgehog
Wingless:GFP
c
PI-PLC
1 2 3 4 5 6 7 8 9 10 11 12
secreted GFP
ApoLII
ApoLII
Fasciclin-1
Fasciclin-1
Klingon
Klingon
AchE
AchE
Connectin
Connectin
d
1 2 3 4 5 6 7 8 9 10 11 12
Wingless
Wingless:GFP
Hedgehog
Hedgehog:HA
larvae
e
1 2 3 4 5 6
ApoLII
Wingless:GFP
Hedgehog:HA
discs
f
S120 P120
1st spin
2nd spin
Hedgehog:HA
g
pre-imm.
anti-ApoLII
ApoLII
Hedgehog
Wingless
ApoLII:Myc
GFPgpi
Hedgehog:HA
h
ApoLII
secreted GFP
pre-imm.
anti-ApoLII
anti-GFP

Figure 8

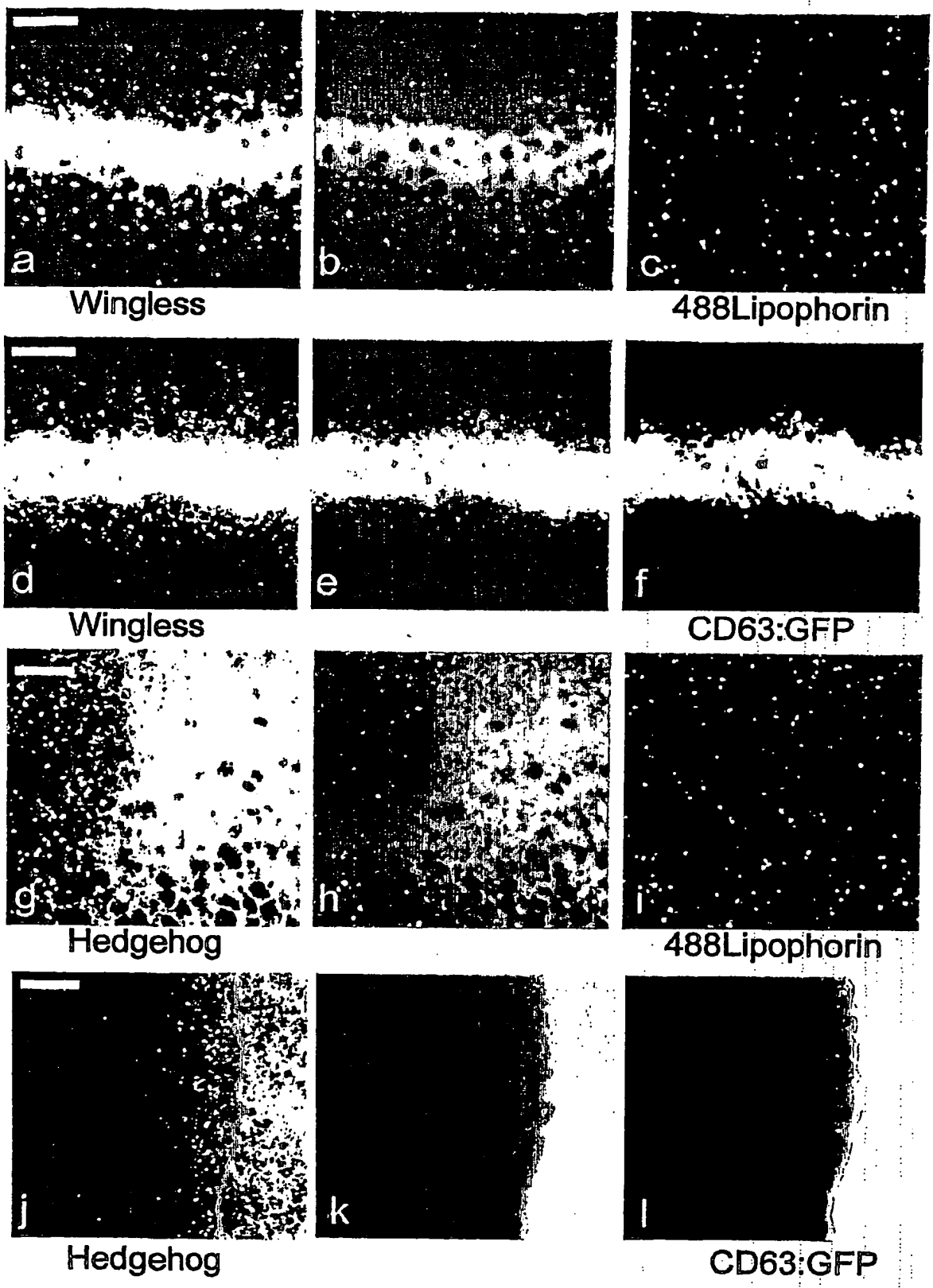

a
b
c
Wingless
488Lipophorin
d
e
f
Wingless
CD63:GFP
g
h
i
Hedgehog
488Lipophorin
j
k
l
Hedgehog
CD63:GFP

Figure 9

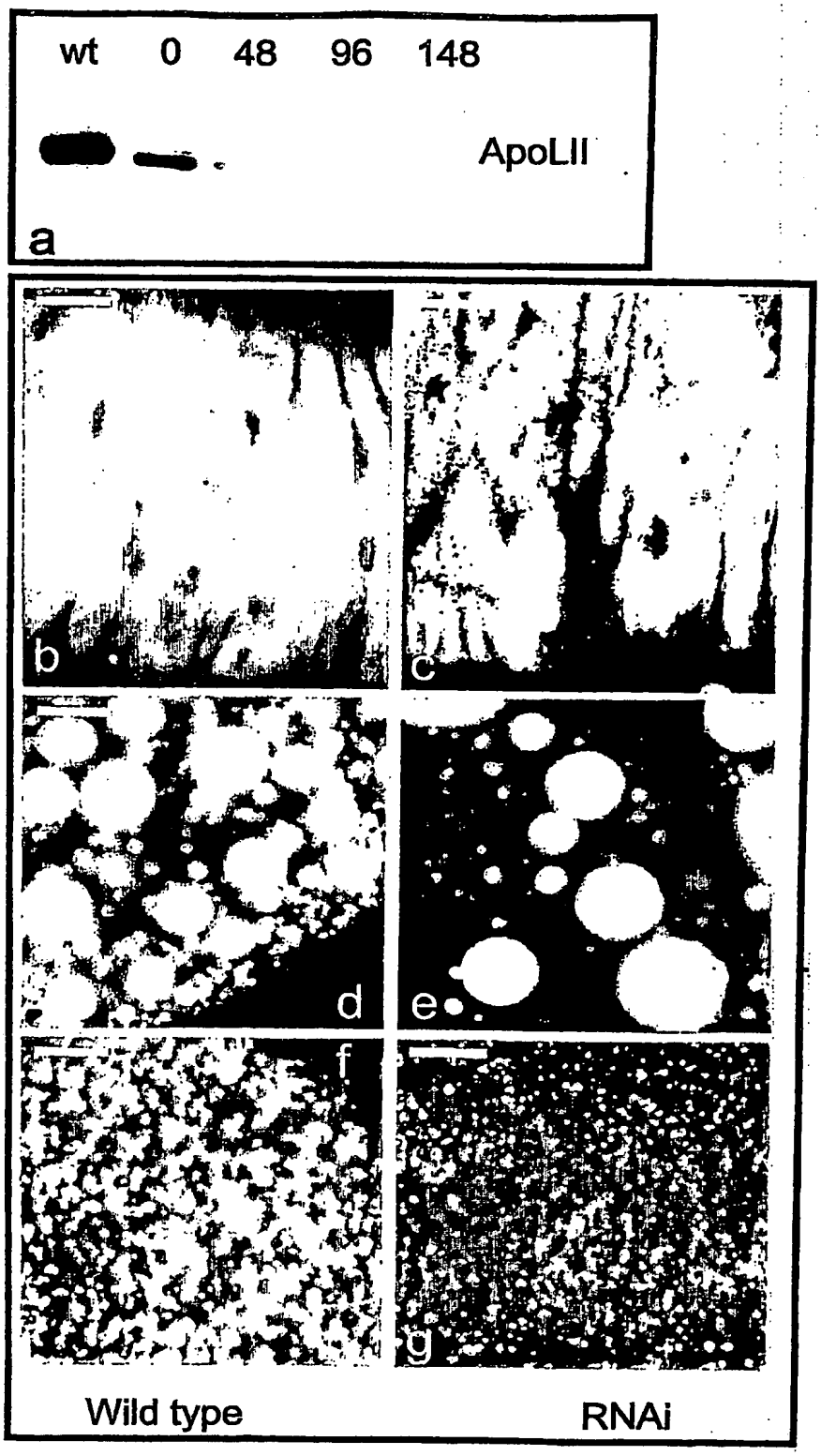


Figure 10

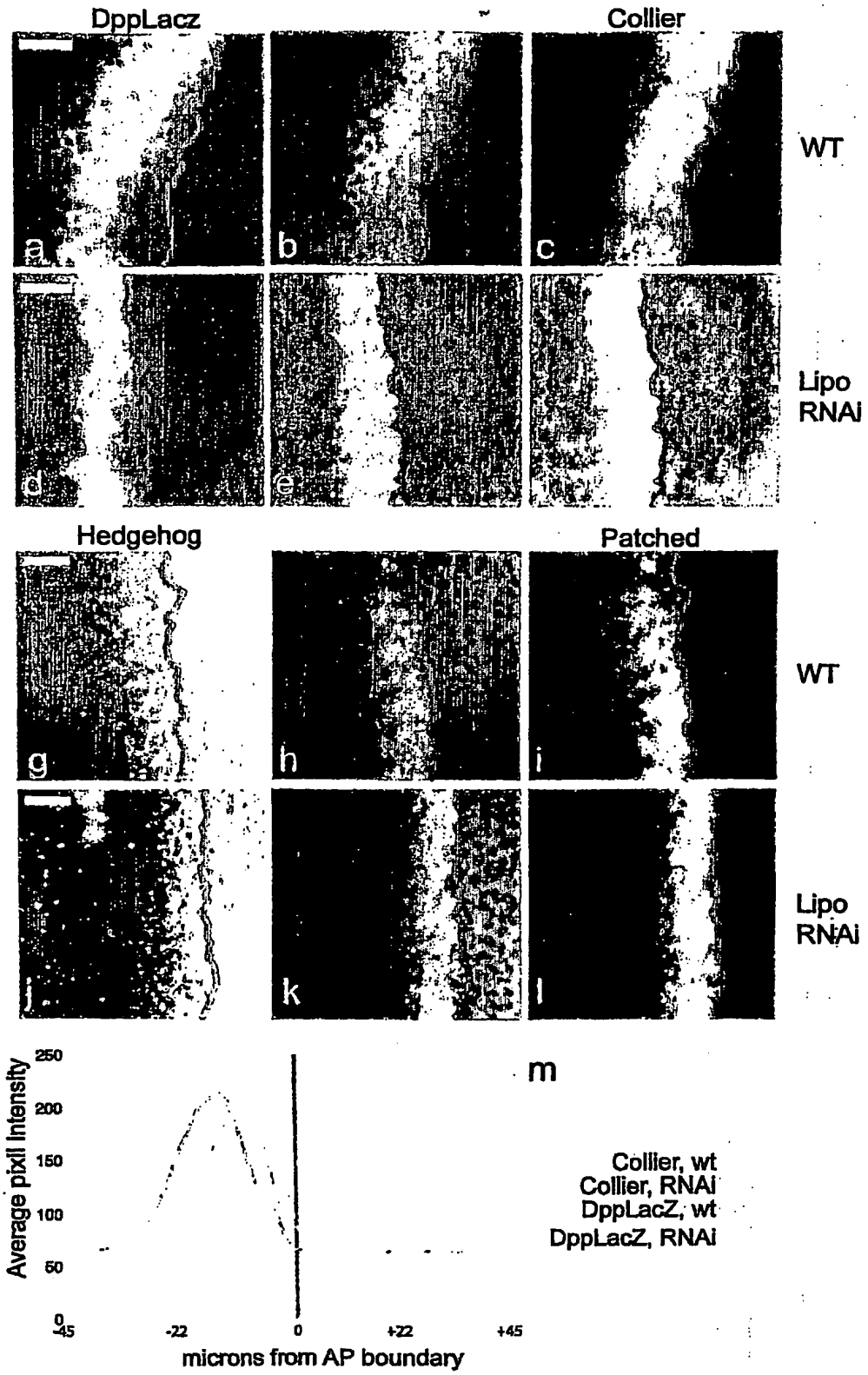

DppLacz
Collier
WT
Lipo RNAi
Hedgehog
Patched
WT
Lipo RNAi
a
b
c
d
e
f
g
h
i
j
k
l
m
Average pixil intensity
250
200
150
100
50
0
-45
-22
0
+22
+45
microns from AP boundary
Collier, wt
Collier, RNAi
DppLacZ, wt
DppLacZ, RNAi

Figure 11

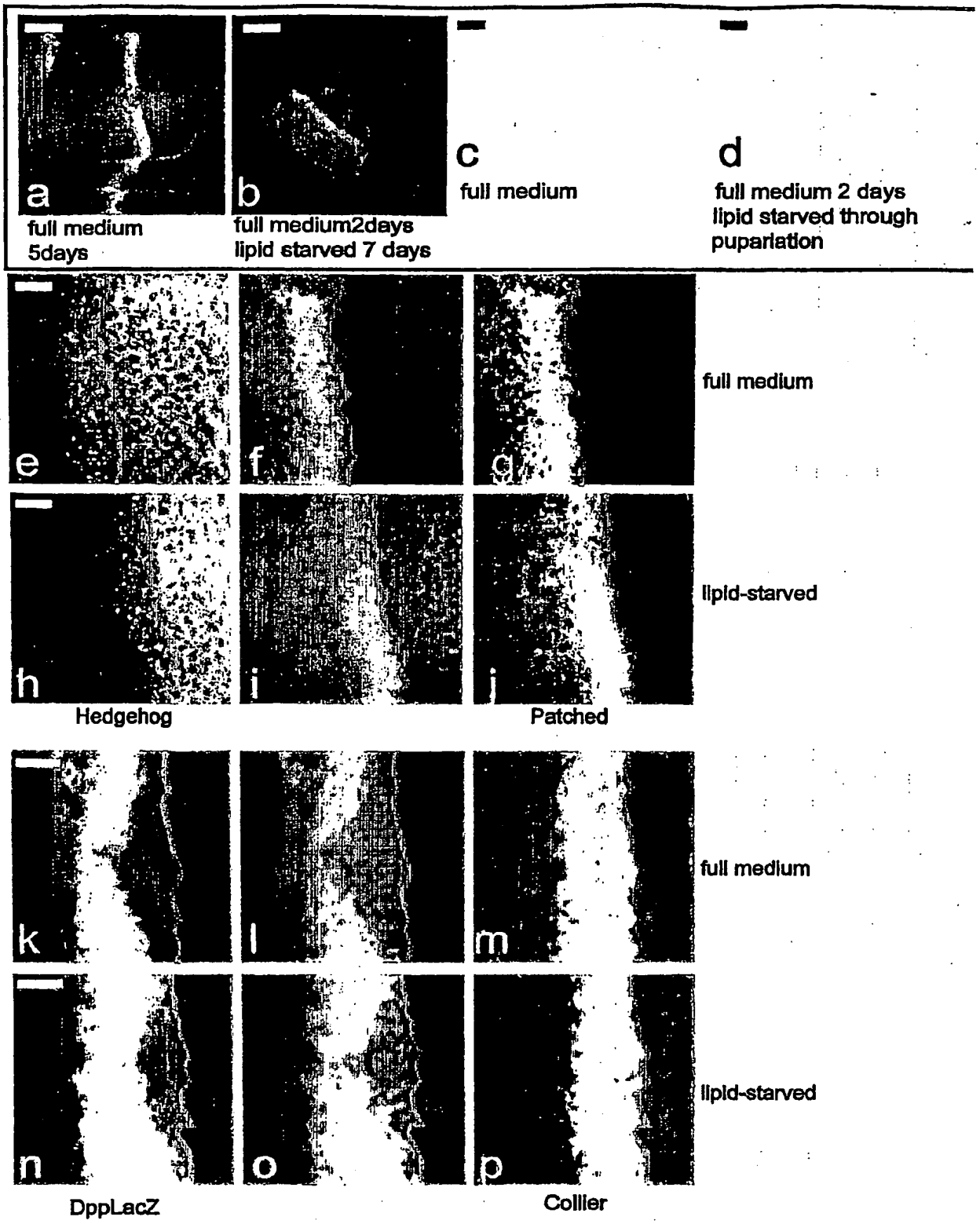
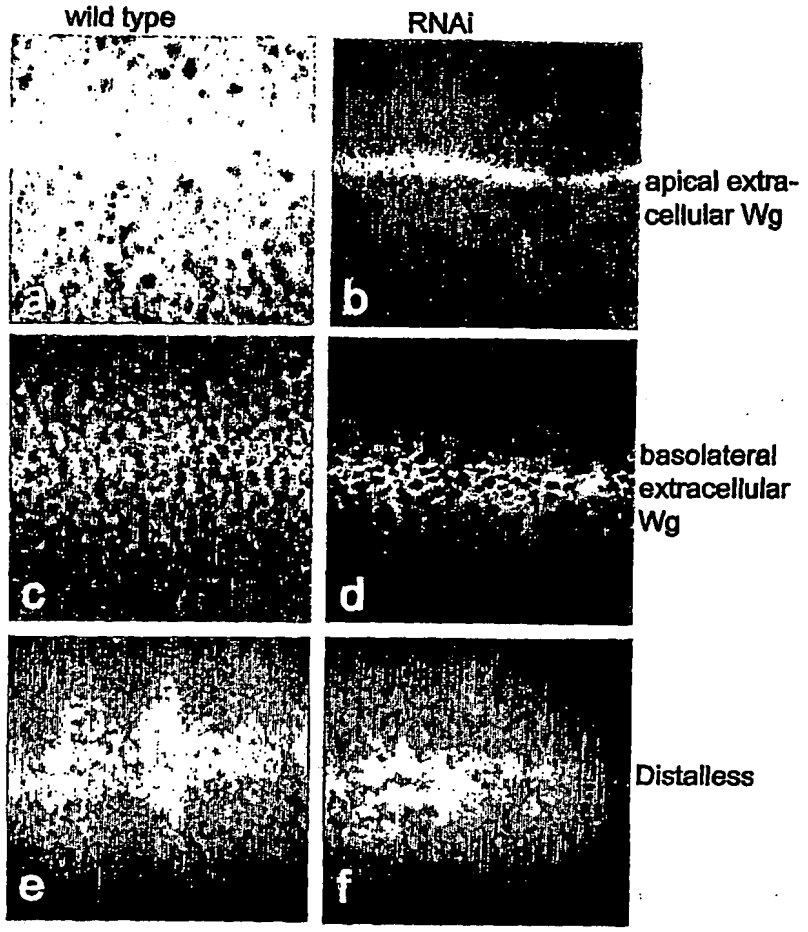

a
full medium
5days
b
full medium2days
lipid starved 7 days
c
full medium
d
full medium 2 days
lipid starved through
puparlation
e
f
g
full medium
h
i
j
lipid-starved
Hedgehog
Patched
k
l
m
full medium
n
o
p
lipid-starved
DppLacZ
Collier

Figure 12


wild type
RNAi
apical extra-cellular Wg
basolateral extracellular Wg
Distalless
a
b
c
d
e
f

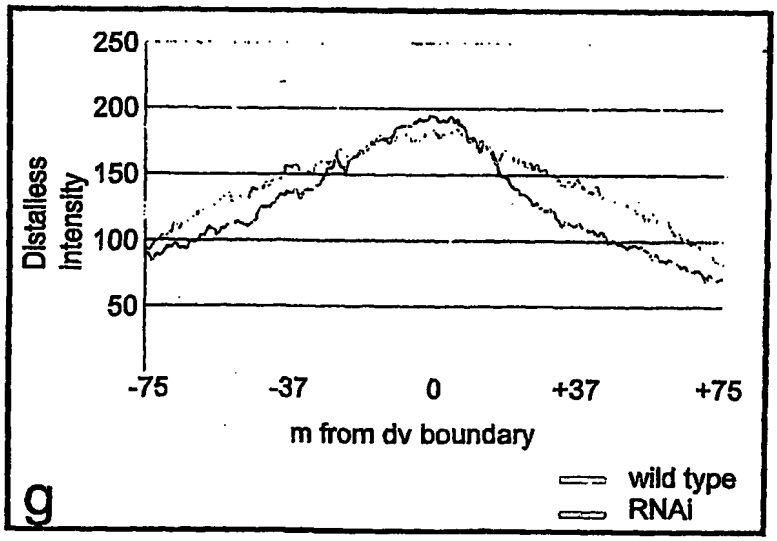

250
200
150
100
50
Distalless intensity
-75
-37
0
+37
+75
m from dv boundary
wild type
RNAi
g

Figure 13

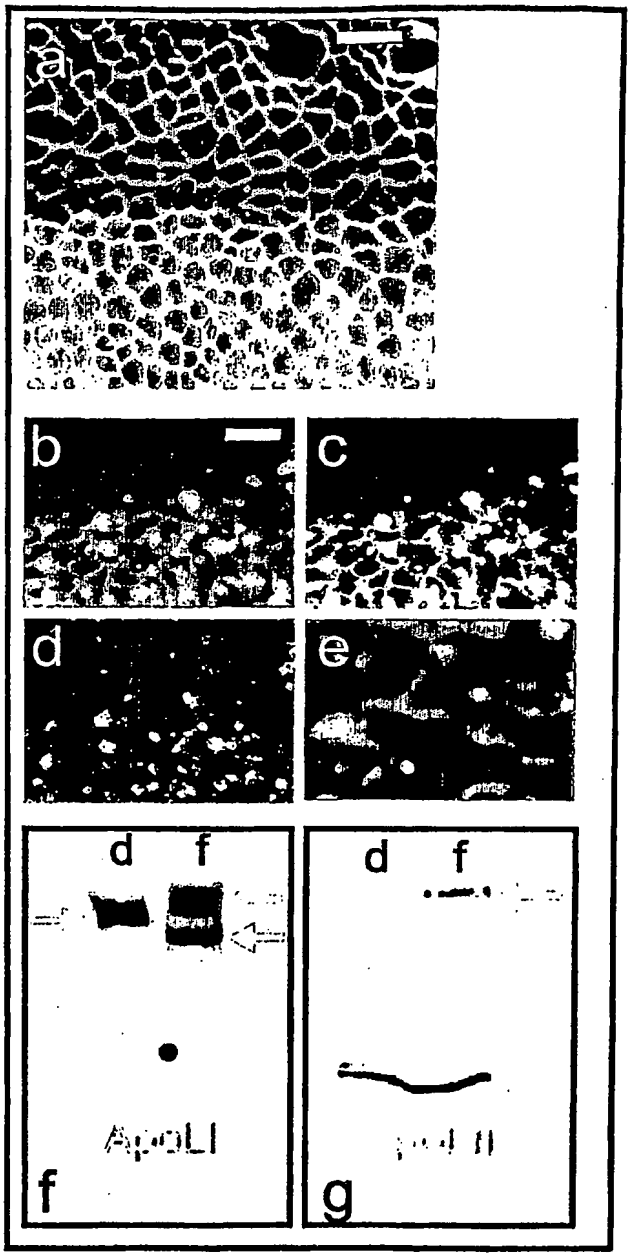


Figure 14

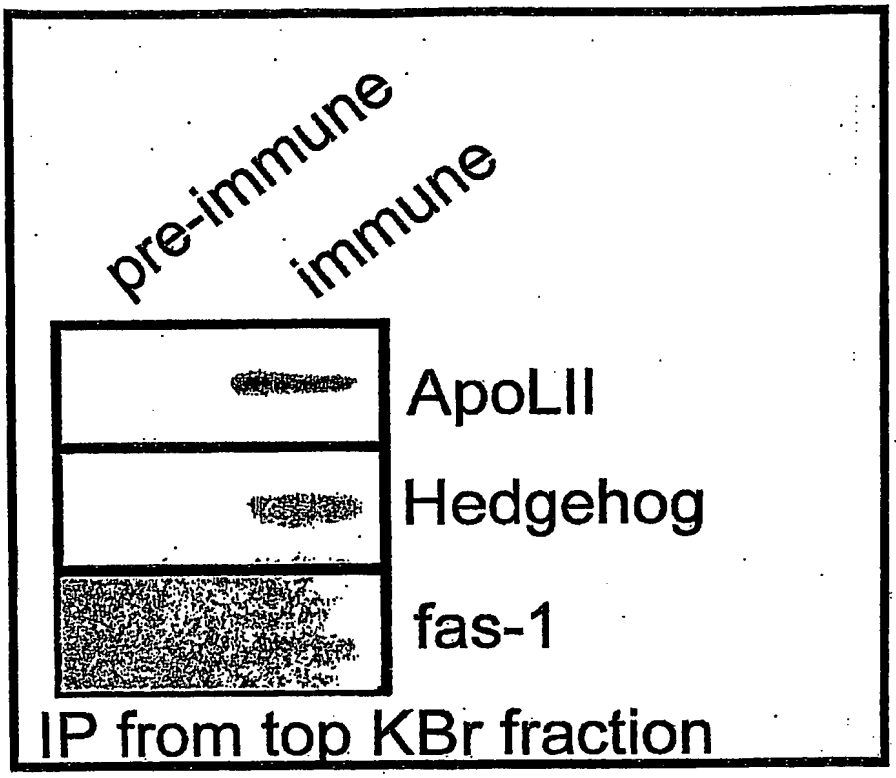

pre-immune
immune
ApoLII
Hedgehog
fas-1
IP from top KBr fraction

Figure 15

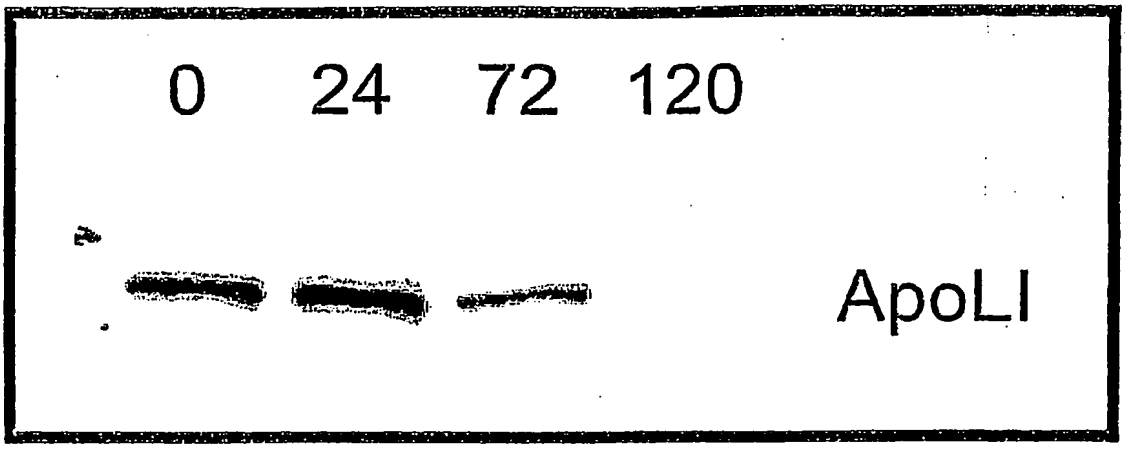


Figure 16

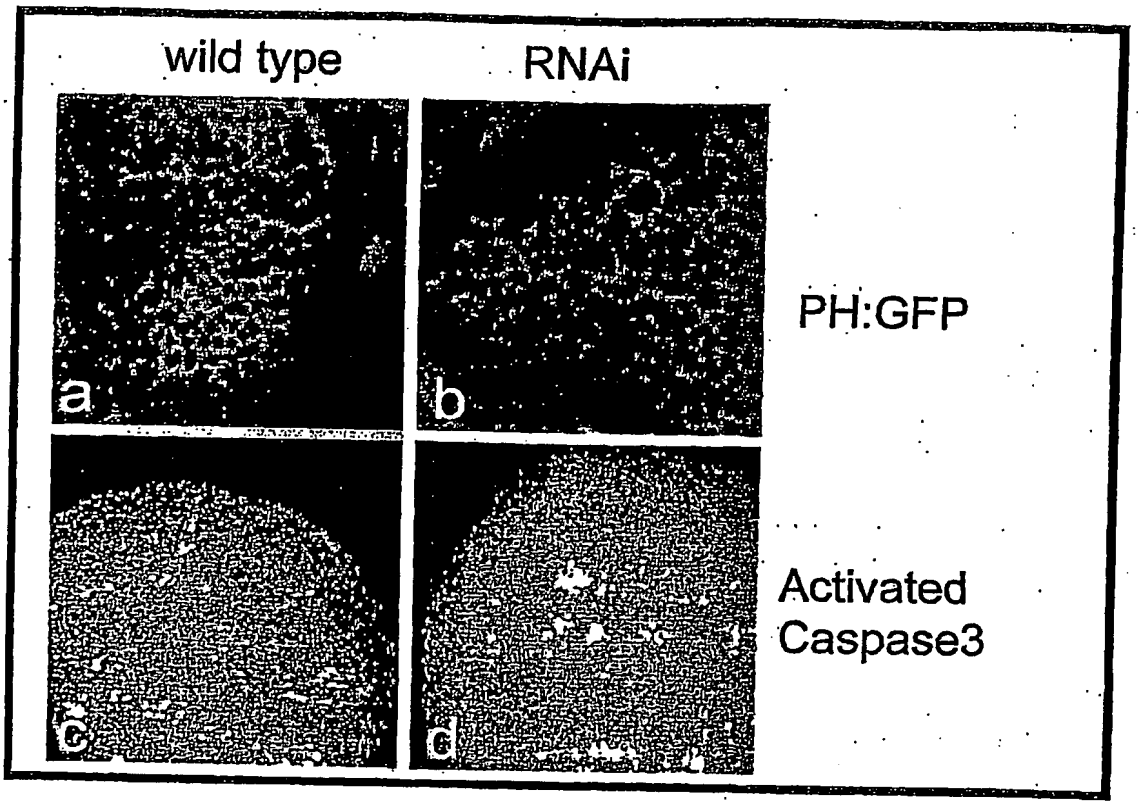


Figure 17

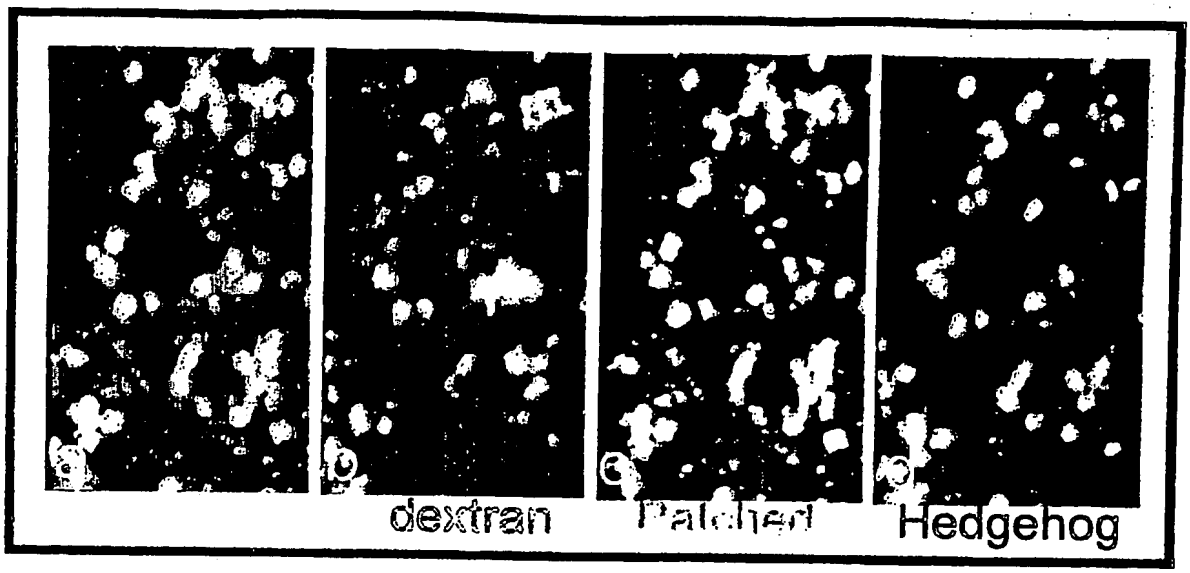


Figure 18

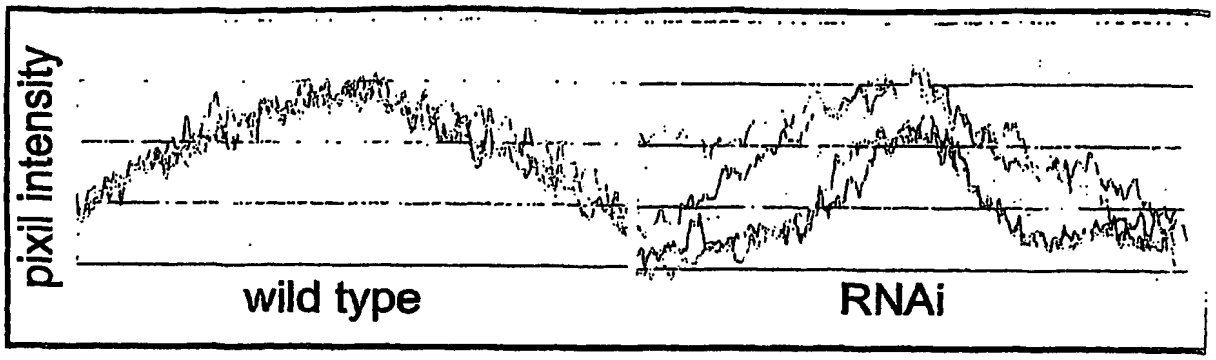
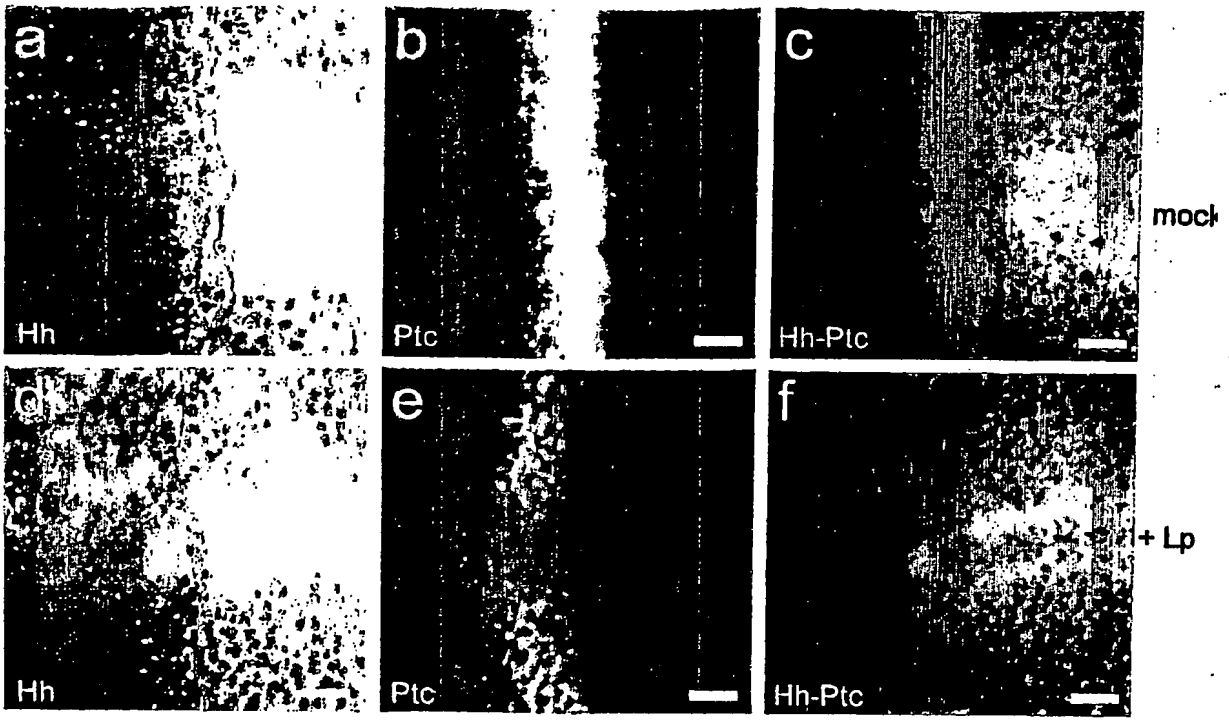


Figure 19



g

	number of Hh-dots	percentage
mock (n=3)	117 +/- 47 (95, 170, 86)	100
+ Lp-particles (n=5)	52 +/- 12 (51, 48, 61, 34, 65)	43.7
ratio mock/+Lp	2.290	

h

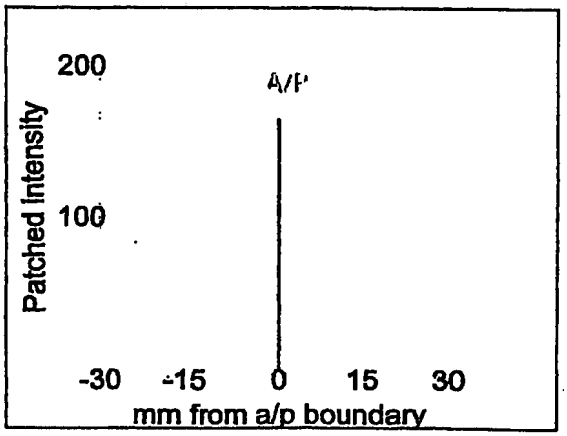
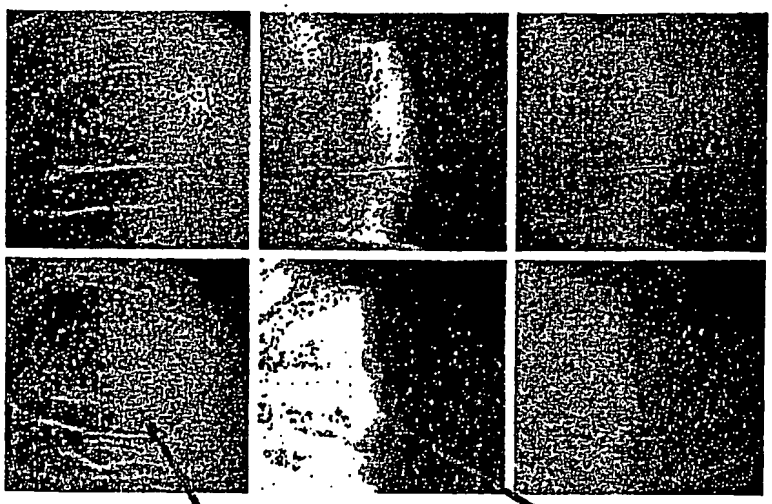
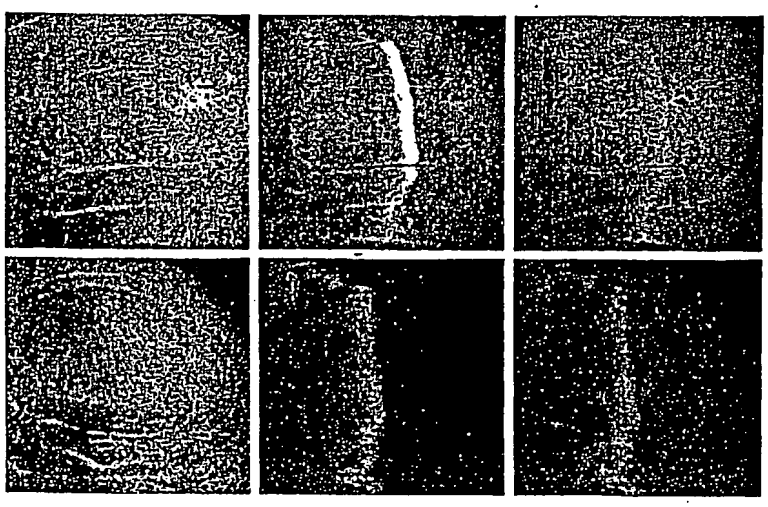


Figure 20

Lipophorin RNAi causes accumulation of a full-length, inactive form of Ci



Although Hedgehog spreads no further, full length Ci accumulates throughout the anterior compartment



Unprocessed Ci does not activate Patched expression

Figure 21

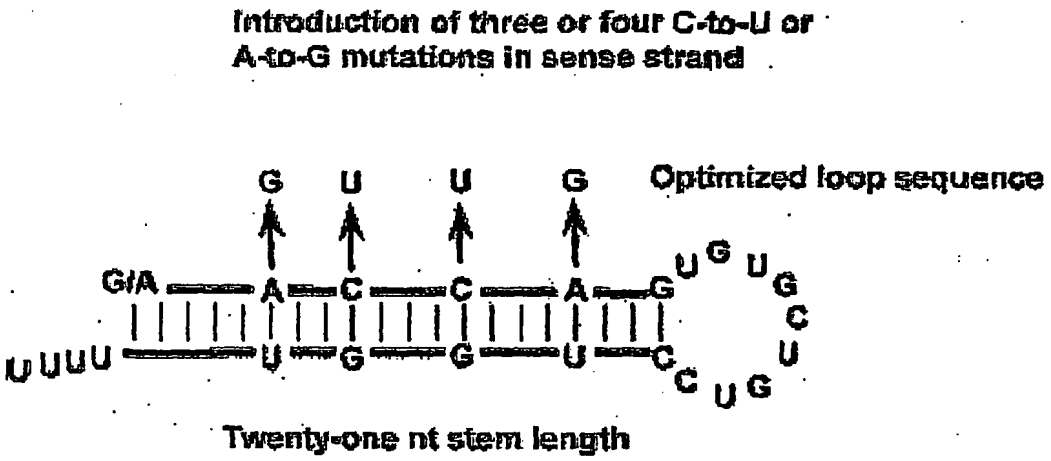

Introduction of three or four C-to-U or
A-to-G mutations in sense strand
Optimized loop sequence
Twenty-one nt stem length

Figure 22

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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13 October 2005 (13.10.2005)

PCT

(10) International Publication Number
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(51) International Patent Classification:
A61P 35/00 (2006.01) *A61K 31/35* (2006.01)
A61K 31/4725 (2006.01) *A61K 31/437* (2006.01)

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(22) International Filing Date: 30 March 2005 (30.03.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
04007732.3 30 March 2004 (30.03.2004) EP

(71) Applicant *(for all designated States except US)*: MAX-PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: EATON, Suzanne [US/DE]; Reger Str. 2, 01309 Dresden (DE). THIELE, Christoph [DE/DE]; Birkenstr. 32, 01474 Dresden (DE). PANAKOVA, Daniela [SK/DE]; Louisenstr. 12, 01099 Dresden (DE). MAROIS, Eric [FR/DE]; Neubertstr. 27, 01307 Dresden (DE). SPRONG, Hein [NL/NL]; Gerrit Jan van der Veenlaan 11, NL-3705 PE Zeist (NL).

(74) Agent: VOSSIUS & Partner; Siebertstrasse 4, 81675 Munich (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
9 February 2006

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2005/094864 A3

(54) Title: TREATMENT OF HEDGEHOG- AND WNT-SECRETING TUMORS WITH INHIBITORS OF LIPOPROTEIN PARTICLE BIOGENESIS

(57) Abstract: This invention relates to the use of an inhibitor of Microsomal Triglyceride Transfer Protein (MTP), HMG-CoA reductase, DGAT and/or ACAT for the preparation of a pharmaceutical composition for the treatment of tumors. In a preferred embodiment, growth and/or progression of the tumor are caused by one or more protein of the Wnt or Hedgehog family. Preferred tumors are esophageal tumor, biliary tract tumor, gastric tumor, pancreatic tumor, malignant melanoma, colorectal tumor, squamous cell carcinoma and cervical tumor.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/003326

A. CLASSIFICATION OF SUBJECT MATTER
A61P35/00 A61K31/4725 A61K31/35 A61K31/437

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/03174 A (BRISTOL-MYERS SQUIBB COMPANY) 29 January 1998 (1998-01-29) page 1, line 1 - line 22 page 40, line 1 - line 29 claims 1-14,25,26	1,8-14, 16-19
X	US 2003/054357 A1 (YOUNG CHARLES ET AL) 20 March 2003 (2003-03-20) claim 3	1,8-14, 16-19
X	US 5 145 839 A (BELJANSKI ET AL) 8 September 1992 (1992-09-08) claims 1-4	1,8-14, 16-19

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2005	08. 12. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ganschow, S

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2005/003326

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 (in part), 8-13 (in part), 14, 16-19 (in part)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1 (in part),8-13 (in part), 14, 16-19 (in part)

   Use of MTP-inhibitors for the preparation of a medicament for the treatment of tumors

---

2. claims: 1 (in part),8-13 (in part), 15-19 (in part)

   Use of an inhibitor of HMG-CoA-reductase for the preparation of a medicament for the treatment of tumors

---

3. claims: 1 (in part), 8-13 (in part), 15-19 (in part)

   Use of an inhibitor of DGAT for the preparation of a medicament for the treatment of tumors

---

4. claims: 1 (in part), 8-13 (in part), 15-19 (in part)

   Use of an inhibitor of ACAT for the preparation of a medicament for the treatment of tumors

---

5. claims: 2, 7-13 (in part), 15-19 (in part)

   Use of an inhibitor of lipoprotein secretion for the preparation of a medicament for the treatment of tumors

---

6. claims: 3, 7-13 (in part), 15-19 (in part)

   Use of an inhibitor of lipoprotein for the preparation of a medicament for the treatment of tumors

---

7. claims: 4, 7-13 (in part), 15-19 (in part)

   Use of an inhibitor of lipoprotein transport for the preparation of a medicament for the treatment of tumors

---

8. claims: 5, 7-13 (in part), 15-19 (in part)

   Use of an inhibitor of lipoprotein association with a target cell for the preparation of a medicament for the treatment of tumors

---

9. claims: 6, 7-13 8in aprt), 16-19 (in part)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Use of an inhibitor of the association of a protein of the Wnt or Hedgehog family with lipoproteins for the preparation of a medicament for the treatment of tumors

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2005/003326

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9803174 A	29-01-1998	AU 712303 B2	04-11-1999
		AU 3600897 A	10-02-1998
		CA 2261162 A1	29-01-1998
		EP 0954313 A1	10-11-1999
		JP 2002513379 T	08-05-2002
US 2003054357 A1	20-03-2003	NONE	
US 5145839 A	08-09-1992	CA 1338325 C	14-05-1996
		DK 310189 A	24-12-1989
		EP 0352147 A2	24-01-1990
		FR 2633182 A1	29-12-1989
		JP 2111725 A	24-04-1990

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(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 June 2006 (15.06.2006)

PCT

(10) International Publication Number
WO 2006/062748 A2

(51) International Patent Classification:
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(21) International Application Number:
PCT/US2005/042648

(22) International Filing Date:
22 November 2005 (22.11.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/633,125	6 December 2004 (06.12.2004)	US
60/659,099	8 March 2005 (08.03.2005)	US
60/699,866	18 July 2005 (18.07.2005)	US

(71) Applicant *(for all designated States except US)*: **RELIANT PHARMACEUTICALS, INC.** [US/US]; 110 Allen Road, Liberty Corner, NJ 07938 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **RONGEN, Roelof, M., L.** [NL/US]; 131 Fairmount Road, East, Caliton, NJ 07930 (US). **BOBOTAS, George** [US/US]; 1245 North Florida Avenue, Tarpon Springs, FL 34689 (US).

(74) Agents: **BERMAN, Richard, J.** et al.; ARENT FOX, PLLC, 1050 Connecticut Avenue NW, Washington, DC 20036 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/062748 A2

(54) Title: OMEGA-3 FATTY ACIDS AND DYSLIPIDEMIC AGENT FOR LIPID THERAPY

(57) Abstract: A method and composition for blood lipid therapy by administering to the subject an effective amount of a dyslipidemic agent and omega-3 fatty acids. The method utilizes a single administration or a unit dosage of a combination of dyslipidemic agent and omega-3 fatty acids for the treatment of patients with hypertriglyceridemia, hypercholesterolemia, mixed dyslipidemia, coronary heart disease (CHD), vascular disease, artherosclerotic disease and related conditions, and the prevention or reduction of cardiovascular and vascular events.

OMEGA-3 FATTY ACIDS AND DYSLIPIDEMIC AGENT FOR LIPID THERAPY

[0001] The present application claims priority from provisional patent application Serial No. 60/633,125, filed December 6, 2004, Serial No. 60/659,099, filed March 8, 2005, and Serial No. 60/699,866, filed July 18, 2005. The disclosure of the provisional applications is hereby incorporated by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to a method utilizing a single administration or a unit dosage of a combination of a dyslipidemic agent and omega-3 fatty acids for the treatment of patients with hypertriglyceridemia, coronary heart disease (CHD), vascular disease, artherosclerotic disease and related conditions, and the prevention or reduction of cardiovascular and vascular events.

BACKGROUND OF THE INVENTION

[0003] In humans, cholesterol and triglycerides are part of lipoprotein complexes in the bloodstream, and can be separated via ultracentrifugation into high-density lipoprotein (HDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) fractions. Cholesterol and triglycerides are synthesized in the liver, incorporated into VLDL, and released into the plasma. High levels of total cholesterol (total-C), LDL-C, and apolipoprotein B (a membrane complex for LDL-C and VLDL-C) promote human atherosclerosis and decreased levels of HDL-C and its transport complex, apolipoprotein A, which are associated with the

development of atherosclerosis. Further, cardiovascular morbidity and mortality in humans can vary directly with the level of total-C and LDL-C and inversely with the level of HDL-C. In addition, researchers have found that non-HDL cholesterol is an important indicator of hypertriglyceridemia, vascular disease, artherosclerotic disease and related conditions. In fact, recently non-HDL cholesterol reduction has been specified as a treatment objective in NCEP ATP III.

[0004] Agents, such as dyslipidemic agents and omega-3 fatty acids, have been used to treat post-myocardial infarction (MI) and adult endogenous hyperlipidemias of hypercholesterolemias and of hypertriglyceridemias, which are generally categorized as "cardiovascular events".

[0005] Dyslipidemic agents commonly include HMG CoA inhibitors (statins), cholesterol absorption inhibitors, niacin and derivatives such as nicotinamide, fibrates, bile acid sequestrants, MTP inhibitors, LXR agonists and/or antagonists and PPAR agonists and/or antagonists.

[0006] Statins, which are 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, have been used to treat hyperlipidemia and arthrosclerosis, for example. Typically, statin monotherapy has been used to treat cholesterol levels, particularly when a patient is not at an acceptable LDL-C level. Statins inhibit the enzyme HMG-CoA reductase, which controls the rate of cholesterol production in the body. Statins lower cholesterol by slowing down the production of cholesterol and by increasing the liver's ability to remove the LDL-cholesterol already in the blood. Accordingly, the major effect of the statins is to lower LDL-cholesterol levels. Statins have been

shown to decrease CHD risk by about one-third. However, statins only appear to have a modest effect on the TG-HDL axis.

[0007] Cholesterol absorption inhibitors, such as ezetimibe and MD-0727, are a class of lipid-lowering compounds that selectively inhibit the intestinal absorption of cholesterol. Ezetimibe acts on brush border of the small intestine and decreases biliary and dietary cholesterol from the small intestine uptake into the enterocytes.

[0008] Cholesteryl ester transfer protein (CETP) inhibitors, such as torcetrapib, inhibit the CETP molecule which, among other things, moves cholesterol from the HDL form to the LDL form. Inhibiting this molecule is, therefore, a promising approach to increasing HDL cholesterol levels.

[0009] Niacin (nicotinic acid or 3-pyridinecarboxylic acid) has previously been used to treat hyperlipidemia and atherosclerosis. Niacin is known to reduce total cholesterol, LDL-C and triglycerides and increase HDL-C. Niacin therapy is also known to decrease serum levels of apolipoprotein B (Apo B), the major protein component of VLDL-C and LDL-C fractions. However, the magnitude of the individual lipid and lipoprotein response from niacin therapy may be influenced by the severity and type of underlying lipid abnormality.

[00010] Fibrates such as fenofibrate, bezafibrate, clofibrate and gemfibrozil, are PPAR-alpha agonists and are used in patients to decrease lipoproteins rich in triglycerides, to increase HDL and to decrease atherogenic-dense LDL. Fibrates are typically orally administered to such patients.

[00011] Fenofibrate or 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoic acid, 1-methylethyl ester, which belongs to the fibrate family, has been known for many years as a medicinal active principle because of its efficacy in

lowering blood triglyceride and cholesterol levels. Fenofibrate is an active principle which is very poorly soluble in water and the absorption of fenofibrate in the digestive tract is limited. A treatment of 40 to 300 mg of fenofibrate per day enables a 20 to 25% reduction of cholesterolemia and a 40 to 50% reduction of triglyceridemia to be obtained.

[00012] Bile acid sequestrants, such as cholestyramine, colestipol and colesevelam, are a class of drugs that binds bile acids, prevents their reabsorption from the digestive system, and reduces cholesterol levels. The usual effect of bile acid sequestrants is to lower LDL-cholesterol by about 10 to 20 percent. Small doses of sequestrants can produce useful reductions in LDL-cholesterol.

[00013] MTP inhibitors, such as implitapide, are known to inhibit the secretion of cholesterol and triglyceride.

[00014] Liver X receptors (LXRs) are "cholesterol sensors" that regulate the expression of genes involved in lipid metabolism in response to specific oxysterol ligands (Repa et al., *Annu. Rev. Cell Dev. Biol.* **16**: 459-481(2000)). LXR agonists and antagonists are potential therapeutic agents for dyslipidemia and atherosclerosis.

[00015] PPAR-gamma agonists, such as the thiazolidinediones pioglitazone and rosiglitazone, have been shown to improve surrogate markers of cardiovascular risk and atherosclerosis. For example, thiazolidinediones decrease C-reactive protein and carotid intima-media thickness. Non-thiazolidinediones, such as tesaglitazar, naviglitizar and muraglitazar, are dual alpha/gamma PPAR agonists. These compounds are used for lowering glucose, insulin, triglycerides and free fatty acids.

[00016] Partial PPAR-gamma agonist/antagonists, such as metaglidasen, are used for the treatment of type II diabetes.

[00017] Marine oils, also commonly referred to as fish oils, are a good source of two omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been found to regulate lipid metabolism. Omega-3 fatty acids have been found to have beneficial effects on the risk factors for cardiovascular diseases, especially mild hypertension, hypertriglyceridemia and on the coagulation factor VII phospholipid complex activity. Omega-3 fatty acids lower serum triglycerides, increase serum HDL-cholesterol, lower systolic and diastolic blood pressure and the pulse rate, and lower the activity of the blood coagulation factor VII-phospholipid complex. Further, omega-3 fatty acids seem to be well tolerated, without giving rise to any severe side effects.

[00018] One such form of omega-3 fatty acid is a concentrate of omega-3, long chain, polyunsaturated fatty acids from fish oil containing DHA and EPA and is sold under the trademark Omacor®. Such a form of omega-3 fatty acid is described, for example, in U.S. Patent Nos. 5,502,077, 5,656,667 and 5,698,594, each incorporated herein by reference.

[00019] Patients with mixed dyslipidemia or hypercholesteremia often present with blood levels of LDL cholesterol greater than 190 mg/dl and triglyceride levels of 200 mg/dl or higher. The use of diet and single-drug therapy does not always decrease LDL cholesterol and triglycerides adequately enough to reach targeted values in patients with mixed dyslipidemia or hypercholesterolemia with or without a concomitant increase

in triglycerides. In these patients, a complementary combination therapy of a dyslipidemic agent and omega-3 fatty acids may be desirable.

[00020] Studies have examined the effects of fish oil and statin therapy. One study found that fish oil and lovastatin increases plasma LDL cholesterol and VLDL cholesterol. Saify *et al.*, *Pakistan J. of Pharm. Sci.* (2003) 16(2): 1-8. Nakamura *et al.* investigated the effects of purified EPA and statins on patients with hyperlipidemia. Patients having baseline triglyceride levels of 2.07 mmol/l (about 182 mg/dl) and already treated with 5-20 mg/day pravastatin or 5 mg/day simvastatin were additionally treated for 3 months with 900 or 1800 mg/day purified (>90%) EPA ethyl ester. It was reported that combination treatment significantly reduced triglyceride levels, and significantly increased HDL-C levels, as compared to baseline monotherapy. LDL-C levels were not reported. Nakamura *et al.*, *Int. J. Clin. Lab Res.* 29:22-25 (1999).

[00021] Davidson *et al.* investigated the effects of marine oil and simvastatin in patients with combined hyperlipidemia. Patients having baseline triglyceride levels of 274.7 mg/dl to 336.8 mg/dl were treated for 12 weeks with 10 mg/day simvastatin and placebo, 7.2 g/day marine oil (SuperEPA® 1200) and placebo, or a combination of simvastatin and SuperEPA®. The content of omega-3 fatty acids in 7.2 g of marine oil used in the study was 3.6 g, with an EPA/DHA ratio of 1.5. Combination treatment was shown to significantly increase HDL-C levels, as compared to marine oil alone. In addition, triglyceride and non-HDL-C levels were significantly reduced with combination treatment. However, non-HDL-C levels were reported to be

reduced less with combination treatment than with simvastatin alone. Davidson *et al.*, *Am J Cardiol* (1997) 80: 797-798.

[00022] Hong *et al.* investigated the effects of fish oil and simvastatin in patients with coronary heart disease and mixed dyslipidemia. Patients having baseline triglyceride levels of 292.8 mg/dl or 269.5 mg/dl were initially treated with 10-20 mg/day simvastatin for 6-12 weeks. Thereafter the patients were treated with simvastatin and placebo or simvastatin and 3 g/day fish oil (Meilekang™). Combined treatment significantly reduced triglyceride levels, as compared to baseline and placebo. In addition, combined treatment numerically increased HDL-C levels, and numerically reduced LDL-C levels, as compared to baseline. However, the changes in HDL-C levels and LDL-C levels were not statistically significant. Hong *et al.*, *Chin. Med. Sci. J.* 19:145-49 (2004).

[00023] Contacos *et al.* investigated the effects of fish oil and pravastatin on patients with mixed hyperlipidemia. Patients having baseline triglyceride levels of 4.6 to 5.5 mmol/l (404 to 483 mg/dl) were initially treated for 6 weeks with 40 mg/day pravastatin, 6 g/day fish oil (Himega™, containing 3 g of omega-3 fatty acids, with an EPA/DHA ratio of 2:1), or placebo. Thereafter, all patients were treated with pravastatin and fish oil for an additional 12 weeks. Initial treatment with pravastatin significantly reduced LDL-C levels. Combined treatment of pravastatin and fish oil also significantly reduced triglyceride and LDL-C levels. However, the addition of fish oil to pravastatin monotherapy resulted in only a numerical increase in LDL-C levels, which was not statistically significant. Treatment with fish oil alone significantly reduced triglyceride levels, but increased LDL-C levels. Combined treatment

for this group significantly reduced LDL-C levels, as compared to fish oil alone (but not as compared to baseline). Contacos *et al.*, *Arterioscl. Thromb.* 13:1755-62 (1993).

[00024] Singer investigated the effects of fish oil and fluvastatin on patients with combined hyperlipidemia. Patients having baseline triglyceride levels of 258 mg/dl were initially treated for two months with 40 mg/day fluvastatin, and thereafter were additionally treated for two months with 3 g/day fish oil (18% EPA and 12% DHA). Thereafter, the patients remained on fluvastatin therapy alone for a final two months. Fluvastatin monotherapy was shown to significantly reduce triglyceride and LDL-C levels, and significantly increase HDL-C levels. Combination treatment significantly reduced triglyceride and LDL-C levels and resulted in an additional numerical reduction of triglyceride and LDL-C levels, as compared to fluvastatin alone. Combination treatment numerically increased HDL-C levels, as compared to monotherapy, although the increase in HDL-C levels with combined treatment was not statistically significant. Singer, *Prost. Leukotr. Ess. Fatty Acids* 72:379-80 (2005).

[00025] Liu *et al.* investigated the effects of fish oil and simvastatin in patients with hyperlipidemia. Patients having baseline triglyceride levels of 1.54 to 1.75 mmol/l (about 136 to 154 mg/dl) were treated for 12 weeks with 10 mg/day simvastatin, 9.2 g/day fish oil (Eskimo-3), or a combination of simvastatin and Eskimo-3. The fish oil contained 18% EPA, 12% DHA, and a total of 38% omega-3 fatty acids. Combined treatment significantly reduced triglyceride and LDL-C levels, and significantly increased HDL-C levels, as compared to baseline, and significantly reduced triglyceride levels as

compared to simvastatin alone. Liu *et al.*, *Nutrition Research* 23 (2003) 1027-1034.

[00026] An additional study concluded that the combined treatment of low-dose pravastatin and fish oil after dinner in post-renal transplantation dislipidemia is more effective to change the lipid profile after renal transplantation. Grekas *et al.*, *Nephron* (2001) 88: 329-333. One article summarizes the combination drug therapies for dyslipidemia, including the combination of statins and 3-7 mg fish oil per day. The study indicates that combination therapy may further augment the reduction of triglyceride, total cholesterol, and apoliprotein E levels, as compared with patients on a statin alone. Alaswad *et al.*, *Curr. Atheroscler. Rep.* (1999) 1: 44-49. In another study, it was found that the combination of dietary fish oil and lovastatin reduces both very low-density lipoprotein (VLDL) and low density lipoprotein (LDL). Huff *et al.*, *Arterosclerosis and Thrombosis*, 12(8): 901-910 (August 1992).

[00027] Additional studies have examined the effects of statins in combination with administration of omega-3 fatty acids and concluded that a diet rich in omega-3 fatty acids increased the cholesterol-lowering effect of simvastatin, counteracted the fasting insulin-elevating effect of simvastatin and did not decrease serum levels of β-carotene and ubiquinol-10. Jula *et al.*, *JAMA* 287 (5) 598-605 (February 6, 2002). Another study showed an increase in thiobarbituric acid-malondialdehyde complex (TBA-MDA) by using EPA and DHA and statins (*e.g.*, simvastatin) did not affect this result. Grundt *et al.*, *Eur. J of Clin. Nutr.* (2003) 57: 793-800.

[00028] U.S. Patent No. 6,720,001 discloses a stabilized pharmaceutical oil-in-water emulsion for delivery of a polyfunctional drug having the drug, an aqueous phase, an oil phase and an emulsifier. Statins are claimed among a list of possible polyfunctional drugs, and fish oil is claimed as one of seven optional components for the oil phase. Moreover, U.S. Patent Application Publication No. 2002/0077317 claims compositions of statins and polyunsaturated fatty acids (PUFAs) (EPA and DHA), while U.S. Patent Application Publication No. 2003/0170643 claims a method of treating a patient, by administering a therapeutic which lowers plasma concentrations of apoB and/or an apoB-containing lipoprotein and/or a component of an atherogenic lipoprotein by stimulating post-ER pre-secretory proteolysis (PERPP) using the combination of fish oils with statins, such as pravastatin, lovastatin, simvastatin, atorvastatin, fluvastatin and cerivastatin.

[00029] Studies have also investigated the effect of statins and concentrated omega-3 fatty acids, specifically the Omacor® omega-3 acids. For example, Hansen *et al.* investigated the effect of lovastatin (40 mg/day) in combination with fish oil concentrate (6 g/day Omacor® omega-3 acids) in patients with hypercholesterolemia. Patients having baseline triglyceride levels of 1.66 mmol/l (about 146 mg/dl) were treated with 6 g/day Omacor® for 6 weeks, followed by 40 mg/day lovastatin for an additional 6 weeks, and a combination of both Omacor® and lovastatin for a final 6 weeks. Lovastatin monotherapy resulted in significant increases in HDL-C levels, and significant decreases in triglyceride and LDL-C levels. After combination treatment, triglyceride and LDL-C levels were further significantly decreased. Hansen *et al.*, *Arteriosclerosis and Thrombosis* 14(2): 223-229 (February 1994).

[00030] Nordoy *et al.* investigated the effect of atorvastatin and omega-3 fatty acids on patients with hyperlipemia. Patients having baseline triglyceride levels of 3.84 mmol/l (about 337 mg/dl) or 4.22 mmol/l (about 371 mg/dl) were treated with 10 mg/day atorvastatin for 5 weeks. Thereafter, for an additional 5 weeks, atorvastatin treatment was supplemented with 2 g/day Omacor® or placebo. Atorvastatin monotherapy, significantly increased HDL-C levels, and triglyceride and LDL-C levels significantly decreased, as compared to baseline. Combination treatment further increased HDL-C levels, as compared to atorvastatin alone. Triglyceride and LDL-C levels numerically further declined slightly with combination treatment, as compared to atorvastatin monotherapy; however, the decrease was insignificant, and the numerical reduction in triglyceride and LDL-C levels was less than with the reduction experienced by the "atorvastatin + placebo" group. The study concluded that the addition of omega-3 fatty acids to statin (*e.g.*, atorvastatin) treatment was an efficient alternative to treating combined hyperlipemia, as the fatty acids further increased HDL-C and reduced systolic blood pressure. Nordoy *et al.*, *Nutr. Metab. Cardiovasc. Dis.* (2001) 11:7-16.

[00031] Salvi *et al.* investigated the effects of Omacor® and simvastatin on patients with familial hypercholesterolemia. Patients having baseline triglyceride levels of 1.355 mmol/l (about 119 mg/dl) and already treated with 20-40 mg/day simvastatin were additionally treated with 6 g/day Omacor® for 4 weeks. It was shown that combination treatment significantly decreased triglyceride and LDL-C levels after 2 weeks, as compared to baseline monotherapy. Salvi *et al.*, *Curr. Ther. Res.* 53:717-21 (1993). Yet another study investigated the effects of omega-3 fatty acids (2 g Omacor® omega-3

acids twice a day) for treating subjects with established CHD and type IIb hyperlipidemia who were already taking simvastatin. The study concluded that the Omacor® omega-3 acids was effective in lowering serum triglyceride levels in patients taking simvastatin. Bhatnagar *et al.*, *Eur. Heart J Supplements* (2001) 4 (Suppl. D): D53-D58.

[00032] Chan *et al.* studied the combined treatment of atorvastatin (40 mg/day) and fish oil (4 Omacor® omega-3 acid capsules orally at night, 4 g/day) on obese, insulin-resistant men with dyslipidemia studied in a fasted state. Patients having baseline triglyceride levels of 1.7 to 2.0 mmol/l (about 150 to 170 mg/dl) were treated for 6 weeks with: 40 mg/day atorvastatin and placebo; 4 g/day Omacor® and placebo; a combination of atorvastatin and Omacor®; or a combination of placebos. Combination treatment significantly decreased triglyceride, non-HDL-C and LDL-C levels, and significantly increased HDL-C, as compared to the placebo group. Chan *et al.*, *Diabetes*, 51: 2377-2386 (Aug. 2002). An additional paper investigated the effects of atorvastatin (40 mg/day) and fish oil (4 g/day Omacor® omega-3 acids at night) on obese men with dyslipidemia and insulin resistance. The treatment groups received a placebo, atorvastatin, the Omacor® omega-3 acids, or a combination thereof at night. The paper concluded that combination treatment of statins and fish oil may be the optimal approach for correcting dyslipidemia in obese men. Chan *et al.*, *Eur. J of Clin. Invest.* (2002) 32: 429-436. Another paper investigated the effects of atorvastatin (40 mg/day) and fish oil (4 g/day Omacor® omega-3 acids at night) on plasma high-sensitivity C-reactive protein concentrations in obese individuals with dyslipidemia. The paper concluded that although fish oil supplementation had no effect on

plasma hs-CRP, the addition of fish oil to statins may further optimize lipid-regulating effects by enhancing a decrease in plasma triglycerides and increase in HDL-C. Chan *et al.*, *Clinical Chemistry* (2002) 48(6): 877-883.

[00033] Nordoy *et al.* investigated the effect of omega-3 fatty acids (3.6 g/day via 4 g/day Omacor® omega-3 acids) and simvastatin (20 mg/day) on patients with combined hyperlipemia. The study concluded that supplementation with the fatty acids reduced hemostatic risk factors and significantly reduced postprandial hyperlipemia. Nordoy *et al.*, *Arterioscler. Thromb. Vasc. Biol.* (2000) 20:259-265.

[00034] Nordoy *et al.* also investigated the efficiency and the safety of treatment with simvastatin and omega-3 fatty acids in patients with hyperlipidemia. Nordoy *et al.*, *J. of Internal Medicine*, 243:163-170 (1998). Patients having baseline triglyceride levels of 2.76 mmol/l (about 243 mg/dl) or 3.03 mmol/l (about 266 mg/dl) were treated for 5 weeks with 20 mg/day simvastatin or placebo, then all patients were treated for an additional 5 weeks with 20 mg/day simvastatin. Thereafter, patients were additionally treated with 4 g/day Omacor® or placebo, for a further 5 weeks. The administration of omega-3 fatty acids with simvastatin resulted in moderate reductions in serum total cholesterol and reduction in triglycerol levels. HDL-C levels slightly decreased, and LDL-C levels slightly increased, with the addition of Omacor®, as compared to the baseline monotherapy.

[00035] Durrington *et al.* examined the effectiveness, safety, and tolerability of a combination of Omacor® omega-3 acids and simvastatin in patients with established coronary heart disease and persisting hypertriglyceridemia. Patients having an average baseline triglyceride levels > 2.3 mmol/l (average

patient serum triglyceride level was 4.6 mmol/l), were treated with 10-40 mg/day simvastatin and 2 g/day Omacor® or placebo, for 24 weeks in a double-blind trial, after which both groups were invited to receive Omacor® for a further 24 weeks in an open study. Combination treatment significantly decreased triglyceride levels within 12 weeks, as compared to baseline monotherapy. In particular, the serum triglyceride levels in patients receiving simvastatin and Omacor® omega-3 acids decreased by 20-30%. In addition, the VLDL cholesterol levels in these patients decreased by 30-40%. LDL-C levels significantly decreased, as compared to baseline monotherapy, only after 48 weeks, although there was a numerical (statistically insignificant) decrease at 12 and 24 weeks. Durrington *et al.*, *Heart*, 85:544-548 (2001).

[00036] U.S. Patent No. 6,096,338, U.S. Patent No. 6,267,985, U.S. Patent No. 6,667,064, U.S. Patent No. 6,720,001, U.S. Patent Application Publication No. 2003/0082215, U.S. Patent Application Publication No. 2004/0052824, WO 99/29300 and WO 2001/021154 disclose compositions, carrier systems and oil-in-water emulsions containing digestible oils or triglycerides with an active ingredient, such as fenofibrate.

[00037] U.S. Patent No. 6,284,268 is directed to self-emulsifying preconcentrate pharmaceutical compositions capable of forming oil-in-water microemulsions or emulsions upon dilution with an aqueous solution, and containing an omega-3 fatty acid oil and a poorly water soluble therapeutic agent, such as a cyclosporin. The '268 patent formulations use a large amount of surfactant (generally higher than 50% w/w, based on the weight of the solvent system), and less than 10% w/w of a hydrophilic solvent system, to achieve the self-emulsifying compositions. Formulation 19 discloses a self-

emulsifying preconcentrate product outside of the scope of the claims of the '268 patent, containing 284 mg of fish oil (about 23% w/w based on the weight of the solvent system, including the fish oil), 663 mg of a surfactant system (about 55% w/w based on the weight of the solvent system), 273 mg of a hydrophilic solvent system (about 22% w/w based on the weight of the solvent system), and 100 mg of fenofibrate. There is no disclosure or suggestion in the '268 patent of a fenofibrate formulation having a solvent system based mainly on fish oil, without the use of a large amount of surfactant. Nor is there any disclosure in the '268 patent regarding administration of the self-emulsifying preconcentrate fenofibrate product to subjects for any treatment. Rather, the '268 patent seemed to use fenofibrate simply to exemplify the solubilizing properties of the self-emulsifying compositions disclosed therein.

[00038] Combinations of omega-3 fatty acids with other fibrates, such as gemfibrozil and clofibrate, have not been shown to produce any dramatic synergistic action in the treatment of hyperlipidemia and hyperlipoproteinemia. See Saify et al., *Pakistan J. of Pharm. Sci.* (2003) 16(2): 1-8; Pennacchiotti et al., *Lipids* (2001) 26(2): 121-127; Wysynski et al., *Human and Experimental Toxicology* (1993) 12: 337-340.

SUMMARY OF THE INVENTION

[00039] There is an unmet need in the art for combination products of dyslipidemic agents and omega-3 fatty acids, in particular a combination product that provides a single administration of concentrated amounts of omega-3 fatty acids and a dyslipidemic agent, for example, in a unit dosage. There is also an unmet need in the art for a method of administration of a single administration or unit dosage product.

[00040] The present invention meets these needs in the art, as well as others, by providing an administration of a unit dosage of a dyslipidemic agent and omega-3 fatty acids that can provide an effective pharmaceutical treatment of coronary heart disease, vascular disease, and related disorders, events, and/or symptoms.

[00041] Some embodiments of the present invention provide for a method of utilizing a combination of a dyslipidemic agent and omega-3 fatty acids in the treatment of hypertriglyceridemia, hypercholesterolemia, mixed dyslipidemia, vascular disease, artherosclerotic disease and related conditions, and the prevention or reduction of cardiovascular and vascular events.

[00042] In a preferred embodiment, the present invention includes methods of blood lipid therapy in a subject comprising administering to the subject an effective amount of a dyslipidemic agent and an omega-3 fatty acid, wherein the subject has a baseline triglyceride level of 200 to 499 mg/dl and wherein after administration to the subject the triglyceride level and a non-HDL-C level of the subject are reduced without increasing LDL-C as compared to treatment with the dyslipidemic agent alone.

[00043] Some embodiments according to the present invention include a method of blood lipid therapy in a subject comprising administering to the subject an effective amount of a dyslipidemic agent and an omega-3 fatty acid, wherein a HDL-C level in the subject is increased and a LDL-C level in the subject is reduced as compared to treatment with the dyslipidemic agent alone.

[00044] In further embodiments, the dyslipidemic agent and the omega-3 fatty acid are administered as a single pharmaceutical composition as a

combination product, for example, a unit dosage, comprising the dyslipidemic agent and the omega-3 fatty acids.

[00045] In preferred embodiments the pharmaceutical compositions comprise Omacor® omega-3 fatty acids, as described in U.S. Patent Nos. 5,502,077, 5,656,667 and 5,698,594. In other preferred embodiments the pharmaceutical compositions comprise omega-3 fatty acids present in a concentration of at least 40% by weight as compared to the total fatty acid content of the composition.

[00046] In still other preferred embodiments the omega-3 fatty acids comprise at least 50% by weight of EPA and DHA as compared to the total fatty acid content of the composition, and the EPA and DHA are in a weight ratio of EPA:DHA of from 99:1 to 1:99, preferably from 1:2 to 2:1.

[00047] In variations of the present invention, the dyslipidemic agent is a statin including, but not limited to, simvastatin, rosuvostatin, pravastatin, atorvastatin, lovastatin and fluvastatin. In preferred embodiments the statin used in combination with omega-3 fatty acids is simvastatin.

[00048] In one aspect of the invention, the combination product is used in the treatment of hypertriglyceridemia, hypercholesterolemia, mixed dyslipidemia, vascular disease, artherosclerotic disease and related conditions, and the prevention or reduction of cardiovascular and vascular events. Yet other embodiments of the present invention are methods for the treatment of hypertriglyceridemia, the reduction of triglycerides and hypertension comprising a combined administration of a dyslipidemic agent and omega-3 fatty acids.

[00049] For example, the methods and compositions of the invention may be used to reduce the LDL-C level of a treated subject. In other embodiments, the triglyceride level of the subject may be reduced. For example, the triglyceride level of the subject may be reduced by at least 10%, preferably about 10% to about 65%, about 15% to about 55%, or about 20% to about 50%, as compared to baseline. In other embodiments, the non-HDL-C level of the subject may be reduced. For example, the non-HDL-C level of the subject may be reduced by at least 10%, preferably about 15% to about 65%, about 25% to about 60% or about 30% to about 55%, as compared to baseline.

[00050] In yet further preferred embodiments of the present invention the triglyceride levels in the serum of subjects prior to the first administration to the subject of a combination of a dyslipidemic agent and omega-3 fatty acid is about 200 to about 499 mg/dl.

[00051] The invention also includes the use of an effective amount of a dyslipidemic agent and an omega-3 fatty acid for the manufacture of a medicament useful for any of the treatment methods indicated herein.

[00052] Other features and advantages of the present invention will become apparent to those skilled in the art upon examination of the following or upon learning by practice of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[00053] The present invention is directed to the utilization of dyslipidemic agents and omega-3 fatty acids, preferably concentrated omega-3 fatty acids, for the treatment of hypertriglyceridemia, hypercholesteremia, mixed dyslipidemia, vascular disease, artherosclerotic disease and related

conditions and the prevention or reduction of cardiovascular and vascular events and a combination product or unit dosage comprising one or more dyslipidemic agents and one or more omega-3 fatty acids.

[00054] In some embodiments, this invention provides a novel combination product for the treatment of hypertriglyceridemia, hypercholesteremia, mixed dyslipidemia, vascular disease, artherosclerotic disease and related conditions, and the prevention or reduction of cardiovascular and vascular events comprising the administration of the combination product to a subject. In a preferred embodiment, the administration comprises omega-3 fatty acids, preferably in the form of the Omacor® omega-3 acids, and a dyslipidemic agent, wherein the omega-3 fatty acids are administered simultaneous to administration of the dyslipidemic agent, *e.g.*, as a single fixed dosage pharmaceutical composition or as separate compositions administered at the same time.

[00055] In other preferred embodiments, the administration comprises omega-3 fatty acids and a dyslipidemic agent, wherein the omega-3 fatty acids are administered apart from the administration of the dyslipidemic agent, but in a concomitant treatment regime. For example, the dyslipidemic agent may be administered weekly with daily intake of omega-3 fatty acids. One skilled in the art with the benefit of the present disclosure will understand that the precise dosage and schedule for the administration of the omega-3 fatty acids and the dyslipidemic agent will vary depending on numerous factors, such as, for example, the route of administration and the seriousness of the condition.

[00056] In preferred embodiments, the present invention includes methods of blood lipid therapy in a subject comprises administering to the subject an effective amount of a dyslipidemic agent and an omega-3 fatty acid, wherein the subject has a baseline triglyceride level of 200 to 499 mg/dl and wherein after administration to the subject the triglyceride level and a non-HDL-C level of the subject are reduced without increasing LDL-C as compared to treatment with the dyslipidemic agent alone.

[00057] In other embodiments, the present invention includes methods of blood lipid therapy in a subject group comprising administering to the subject group an effective amount of a dyslipidemic agent and an omega-3 fatty acid, wherein the subject group has a baseline triglyceride level of 200 to 499 mg/dl and wherein after administration to the subject group the triglyceride level and a non-HDL-C level of the subject group are reduced in a statistically significant amount as compared to a control group treated with the dyslipidemic agent alone without increasing LDL-C in a statistically significant amount as compared to the control group treated with the dyslipidemic agent alone.

[00058] Still other embodiments according to the present invention include a method of blood lipid therapy in a subject comprising administering to the subject an effective amount of a dyslipidemic agent and an omega-3 fatty acid, wherein a HDL-C level in the subject is increased and a LDL-C level in the subject is reduced as compared to treatment with the dyslipidemic agent alone. Preferably, the HDL-C level is increased by at least 5%, preferably about 5% to about 30%, preferably by at least 10%, more preferably by at least 15%.

[00059] The phrase "compared to treatment with dyslipidemic agent alone" can refer to treatment in the same subject, or treatment of a comparable subject (i.e., a subject within the same class with respect to a particular blood protein, cholesterol or triglyceride level) in a different treatment group.

[00060] The present invention may incorporate now known or future known dyslipidemic agents in an amount generally recognized as safe. Preferred dyslipidemic agents include HMG CoA inhibitors including statins, cholesterol absorption inhibitors such as but not limited to ezetimibe, niacin and derivatives such as nicotinamide, CETP inhibitors such as but not limited to torcetrapib, fibrates such as but not limited to fenofibrate, bezafibrate, clofibrate and gemfibrozil, bile acid sequestrants such as but not limited to cholestyramine, cholestipol and colesevelam, MTP inhibitors such as but not limited to those disclosed in WO 00/38725 and Science, 282, 23 October 1998, pp. 751-754, herein incorporated by reference, LXR agonists and/or antagonists, and PPAR agonists and antagonists (such as but not limited to PPAR-alpha, PPAR-gamma, PPAR-delta, PPAR-alpha/gamma, PPAR-gamma/delta, PPAR-alpha/delta, and PPAR-alpha/gamma/delta agonists, antagonists and partial agonists and/or antagonists) such as but not limited to the thiazolidinediones, the non-thiazolidinediones and metaglidasen. There are currently six statins that are widely available: atorvastatin, rosuvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin. A seventh statin, cerivastatin, has been removed from the U.S. market at the time of this writing. However, it is conceivable to one skilled in the art that cerivastatin may be used in conjunction with some embodiments of the present invention if cerivastatin is ultimately determined to be safe and effective.

[00061] Generally, the effect of the dyslipidemic agent is dose dependent, i.e., the higher the dose, the greater the therapeutic affect. However, the effect of each dyslipidemic agent is different, and therefore the level of therapeutic effect of one dyslipidemic agent cannot be necessarily be directly correlated to the level of therapeutic effects of other dyslipidemic agents. However, those of ordinary skill in the art would understand the correct dosage to be given to a particular subject, based on experience and the seriousness of the condition.

[00062] As used herein, the term "omega-3 fatty acids" includes natural or synthetic omega-3 fatty acids, or pharmaceutically acceptable esters, derivatives, conjugates (see, e.g., Zaloga et al., U.S. Patent Application Publication No. 2004/0254357, and Horrobin et al., U.S. Patent No. 6,245,811, each hereby incorporated by reference), precursors or salts thereof and mixtures thereof. Examples of omega-3 fatty acid oils include but are not limited to omega-3 polyunsaturated, long-chain fatty acids such as a eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and α-linolenic acid; esters of omega-3 fatty acids with glycerol such as mono-, di- and triglycerides; and esters of the omega-3 fatty acids and a primary, secondary or tertiary alcohol such as fatty acid methyl esters and fatty acid ethyl esters. Preferred omega-3 fatty acid oils are long-chain fatty acids such as EPA or DHA, triglycerides thereof, ethyl esters thereof and mixtures thereof. The omega-3 fatty acids or their esters, derivatives, conjugates, precursors, salts and mixtures thereof can be used either in their pure form or as a component of an oil such as fish oil, preferably purified fish oil concentrates. Commercial examples of omega-3 fatty acids suitable for use in the invention include

Incromega F2250, F2628, E2251, F2573, TG2162, TG2779, TG2928, TG3525 and E5015 (Croda International PLC, Yorkshire, England), and EPAX6000FA, EPAX5000TG, EPAX4510TG, EPAX2050TG, K85TG, K85EE, K80EE and EPAX7010EE (Pronova Biocare a.s., 1327 Lysaker, Norway).

[00063] Preferred compositions include omega-3 fatty acids as recited in U.S. Patent Nos. 5,502,077, 5,656,667 and 5,698,694, which are hereby incorporated herein by reference in their entireties.

[00064] Another preferred composition includes omega-3 fatty acids present in a concentration of at least 40% by weight, preferably at least 50% by weight, more preferably at least 60% by weight, still more preferably at least 70% by weight, most preferably at least 80% by weight, or even at least 90% by weight. Preferably, the omega-3 fatty acids comprise at least 50% by weight of EPA and DHA, more preferably at least 60% by weight, still more preferably at least 70% by weight, most preferably at least 80%, such as about 84% by weight. Preferably the omega-3 fatty acids comprise about 5 to about 100% by weight, more preferably about 25 to about 75% by weight, still more preferably about 40 to about 55% by weight, and most preferably about 46% by weight of EPA. Preferably the omega-3 fatty acids comprise about 5 to about 100% by weight, more preferably about 25 to about 75% by weight, still more preferably about 30 to about 60% by weight, and most preferably about 38% by weight of DHA. All percentages above are by weight as compared to the total fatty acid content in the composition, unless otherwise indicated.

[00065] The EPA:DHA ratio may be from 99:1 to 1:99, preferably 4:1 to 1:4, more preferably 3:1 to 1:3, most preferably 2:1 to 1:2. The omega-3 fatty acids may comprise pure EPA or pure DHA.

[00066] The omega-3 fatty acid composition optionally includes chemical antioxidants, such as alpha tocopherol, oils, such as soybean oil and partially hydrogenated vegetable oil, and lubricants such as fractionated coconut oil, lecithin and a mixture of the same.

[00067] The most preferred form of omega-3 fatty acids is the Omacor® omega-3 acid (K85EE, Pronova Biocare A.S., Lysaker, Norway) and preferably comprises the following characteristics (per dosage form):

Test	Minimum Value	Maximum Value
Eicosapentaenoic acid C20:5	430 mg/g	495 mg/g
Docosahexaenoic acid C22:6	347 mg/g	403 mg/g
EPA and DHA	800 mg/g	880 mg/g
Total n-3 fatty acids	90 % (w/w)	

[00068] The combination product of a dyslipidemic agent and concentrated omega-3 fatty acids may be administered in a capsule, a tablet, a powder that can be dispersed in a beverage, or another solid oral dosage form, a liquid, a soft gel capsule or other convenient dosage form such as oral liquid in a capsule, as known in the art. In some embodiments, the capsule comprises a hard gelatin. The combination product may also be contained in a liquid suitable for injection or infusion.

[00069] The active ingredients of the present invention may also be administered with a combination of one or more non-active pharmaceutical ingredients (also known generally herein as "excipients"). Non-active ingredients, for example, serve to solubilize, suspend, thicken, dilute, emulsify, stabilize, preserve, protect, color, flavor, and fashion the active

ingredients into an applicable and efficacious preparation that is safe, convenient, and otherwise acceptable for use. Thus, the non-active ingredients may include coilloidal silicon dioxide, crospovidone, lactose monohydrate, lecithin, microcrystalline cellulose, polyvinyl alcohol, povidone, sodium lauryl sulfate, sodium stearyl fumarate, talc, titanium dioxide and xanthum gum.

[00070] Excipients include surfactants, such as propylene glycol monocaprylate, mixtures of glycerol and polyethylene glycol esters of long fatty acids, polyethoxylated castor oils, glycerol esters, oleoyl macrogol glycerides, propylene glycol monolaurate, propylene glycol dicaprylate/dicaprate, polyethylene-polypropylene glycol copolymer, and polyoxyethylene sorbitan monooleate, cosolvents such ethanol, glycerol, polyethylene glycol, and propylene glycol, and oils such as coconut, olive or safflower oils. The use of surfactants, cosolvents, oils or combinations thereof is generally known in the pharmaceutical arts, and as would be understood to one skilled in the art, any suitable surfactant may be used in conjunction with the present invention and embodiments thereof.

[00071] The combination product of a dyslipidemic agent and concentrated omega-3 fatty acids is aided by the solubility of the dyslipidemic agent in the omega-3 fatty acid oil. Thus, the combination product does not require high amounts of solubilizers, such as surfactants, cosolvents, oils or combinations thereof. Preferably, the active ingredients are administered without the use of large amounts of solubilizers (other than the omega-3 fatty acid oil). In preferred embodiments, if present at all, solubilizers other than the omega-3 fatty acid oil are present in amounts of less than 50% w/w based on the total

weight of the solvent system in the dosage form(s), preferably less than 40%, more preferably less than 30%, even more preferably less than 20%, still more preferably less than 10% and most preferably less than 5%. In some embodiments, the solvent system contains no solubilizers other than the omega-3 fatty acid oil. As used herein, "solvent system" includes the omega-3 fatty acid oil. In other preferred embodiments, the weight ratio of omega-3 fatty acid oil to other solubilizer is at least 0.5 to 1, more preferably at least 1 to 1, even more preferably at least 5 to 1, and most preferably at least 10 to 1.

[00072] In other preferred embodiments, if present at all, the amount of hydrophilic solvent used in the solvent system is less than 20% w/w based on the total weight of the solvent system in the dosage form(s), more preferably less than 10%, and most preferably less than 5%. In certain embodiments, the amount of hydrophilic solvent used in the solvent system is between 1 and 10% w/w.

[00073] In preferred embodiments, omega-3 fatty acid oil is present in amounts of at least 30% w/w based on the total weight of the solvent system in the dosage form(s), more preferably at least 40%, even more preferably at least 50%, and most preferably at least 60%. In certain embodiments, the amount can be at least 70%, at least 80% or at least 90%.

[00074] The dosage form is stable at room temperature (about 23°C to 27°C) for a period of at least one month, preferably at least six months, more preferably at least one year, and most preferably at least two years. By "stable", applicants mean that the solubilized dyslipidemic agent does not come out of solution to any appreciable degree, for example, in amounts of less than 10%, preferably less than 5%.

[00075] The concentrated omega-3 fatty acids can be administered in a daily amount of from about 0.1 g to about 10 g, more preferably about 1 g to about 6 g, and most preferably from about 2 g to about 4 g.

[00076] The dyslipidemic agent may be administered in an amount more than, equal to or less than the conventional full-strength dose as a single-administered product. For example, the dyslipidemic agent may be administered in an amount of from 10-100%, preferably about 25-100%, most preferably about 50-80%, of the conventional full-strength dose as a single-administered product. In one embodiment of the present invention, the statin can generally be present in an amount from about 0.5 mg to 80 mg, more preferably from about 1 mg to about 40 mg, and most preferably from about 5 mg to about 20 mg, per gram of omega-3 fatty acid. The daily dose may range from about 2 mg to about 320 mg, preferably about 4 mg to about 160 mg.

[00077] In some variations of the present invention, the combination of dyslipidemic agent and the omega-3 fatty acids is formulated into a single administration or unit dosage. In preferred embodiments, a statin is utilized selected from the following group: atorvastatin, rosuvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin.

[00078] Pravastatin, which is known in the market as Pravachol® manufactured by Bristol-Myers Squibb, Princeton, NJ, is hydrophilic. Pravastatin is best absorbed without food, i.e., an empty stomach. The dosage of pravastatin, in the combined administration of concentrated omega-3 fatty acids is preferably from 2.5 to 80 mg, preferably 5 to 60, and more preferably from 10 to 40 mg per dosage of concentrated omega-3 fatty acids.

In one variation, the combination product using pravastatin is taken at or around bedtime, *e.g.*, 10 pm.

[00079] Lovastatin, which is marketed under the name Mevacor® by Merck, Whitehouse Station, NJ, is hydrophobic. Unlike pravastatin, lovastatin should be taken with meals and accordingly, in some embodiments, the combination product of concentrated omega-3 fatty acids and lovastatin should be taken with food. The dosage of lovastatin, in the combined administration of concentrated omega-3 fatty acids is preferably from 2.5 to 100 mg, preferably 5 to 80 mg, and more preferably from 10 to 40 mg per dosage of concentrated omega-3 fatty acids.

[00080] Simvastatin, which is marketed under the name Zocor® by Merck, Whitehouse Station, NJ, is hydrophobic. The dosage of simvastatin, in the combined administration of concentrated omega-3 fatty acids is preferably from 1 to 80 mg per day, preferably 2 to 60 mg, and more preferably from 5 to 40 mg per dosage of concentrated omega-3 fatty acids.

[00081] Atorvastatin, which is marketed under the name Lipitor® by Pfizer, New York, NY, is hydrophobic and is known as a synthetic statin. The dosage of atorvastatin, in the combined administration of concentrated omega-3 fatty acids is preferably from 2.5 to 100 mg, preferably 5 to 80 mg, and more preferably from 10 to 40 mg per dosage of concentrated omega-3 fatty acids.

[00082] Fluvastatin, which is marketed under the name Lescol® by Novartis, New York, NY, is hydrophilic and is known as a synthetic statin. The dosage of fluvastatin, in the combined administration of concentrated omega-3 fatty acids is from 5 to 160 mg, preferably 10 to 120 mg, and more preferably from 20 to 80 mg per dosage of concentrated omega-3 fatty acids.

[00083] Rosuvastatin is marketed under the name Crestor® by Astra Zeneca, Wilmington, DE. The dosage of rosuvastatin, in the combined administration of concentrated omega-3 fatty acids is from 1 to 80 mg, preferably 2 to 60 mg, and more preferably from 5 to 40 mg per dosage of concentrated omega-3 fatty acids.

[00084] The daily dosages of dyslipidemic agent and concentrated omega-3 fatty acids can be administered together in from 1 to 10 dosages, with the preferred number of dosages from 1 to 4 times a day, most preferred 1 to 2 times a day. The administration is preferably oral administration, although other forms of administration that provides a unit dosage of dyslipidemic agent and concentrated omega-3 fatty acids may be used.

[00085] In some embodiments, the formulations of the present invention allow for improved effectiveness of each active ingredient, with one or both administered as a conventional full-strength dose, as compared to the formulations in the prior art. In other embodiments, the formulations of the present invention may allow for reduced dosages of dyslipidemic agent and/or omega-3 fatty acids, as compared to the formulations in the prior art, while still maintaining or even improving upon the effectiveness of each active ingredient.

[00086] The present combination of a dyslipidemic agent and concentrated omega-3 fatty acids may allow for a greater effect than any expected combined or additive effect of the two drugs alone. Moreover, the combined or additive effect of the two drugs may depend on the initial level of triglycerides in the blood of a subject. For example, the triglyceride level of a subject is generally as normal if less than 150 mg/dL, borderline to high if

within about 150-199 mg/dL, high if within about 200-499 mg/dL and very high if 500 mg/dL or higher. The present invention may be used to reduce the triglyceride level of a "very high" down to a "high" or "borderline to high" in less than 48 weeks, preferably within 24 weeks, more preferably within 12 weeks, and most preferably within 6 weeks, 4 weeks or 2 weeks. The present invention may also be used to reduce the triglyceride level of a "high" down to a "borderline to high" or "normal" in less than 48 weeks, preferably within 24 weeks, more preferably within 12 weeks, and most preferably within 6 weeks, 4 weeks or 2 weeks.

[00087] Thus, the combined treatment of the two active ingredients, separately or through the novel combination product of the present invention, may cause an unexpected increase in effect of the active ingredients that allows increased effectiveness with standard dosages or maintained effectiveness with reduced dosages of the two active ingredients. It is well accepted in practice that an improved bioavailability or effectiveness of a drug or other active ingredient allows for an appropriate reduction in the daily dosage amount. Any undesirable side effects may also be reduced as a result of the lower dosage amount and the reduction in excipients (*e.g.*, surfactants).

[00088] The utilization of a single administration of a combination of a dyslipidemic agent and concentrated omega-3 fatty acids overcomes the limitations of the prior art by improving the efficacy of the dyslipidemic agent and the concentrated omega-3 fatty acids, and allows for a treatment with improved effectiveness and less excipients than in multiple administrations of omega-3 fatty acids and dyslipidemic agents.

[00089] The administration of a combination of dyslipidemic agent and concentrated omega-3 fatty acids achieves results that are highly advantageous and beneficial to the pharmaceutical and medicinal arts. The increased efficacy of the combined treatment and combination product allows for a novel and more efficient pharmaceutical treatment for hypertriglyceridemia, hypercholesterolemia, mixed dyslipidemia, vascular disease, artherosclerotic disease and related conditions, the prevention or reduction of cardiovascular and vascular events.

EXAMPLES

[00090] The effect of 4 grams per day of Omacor® omega-3 fatty acids on the lipid parameters, i.e. triglyceride levels (TG), total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoprotein (VLDL), of patients with different baseline TG levels has been evaluated. The Omacor® omega-3 fatty acids were supplied as a liquid-filled gel capsule for oral administration. Each one gram capsule of Omacor® contained at least 900 mg of ethyl esters of omega-3 fatty acids, which comprises predominantly eicosapentaenoic acid (EPA) (about 465 mg) and docosahexaenoic acid (DHA) (about 375 mg). As shown in Table 1, the effectiveness of Omacor® omega-3 fatty acids is dependent on the baseline TG levels of the treated of patients.

Table 1. Percent Change in Lipid Parameters in Patients after administration of Omacor® as Monotherapy

Baseline TG (mg/dL)	TG	Total cholesterol	HDL	LDL	VLDL	Non-HDL
0-199	-22.5	3.5	5.2	10.7	-31.6	3.8
200-299	-23.0	0.2	7.3	5.9	-21.2	-0.5
300-399	-26.1	-1.1	6.1	9.9	-22.3	-1.2
400-499	-25.9	-4.7	12.6	18.9	-8.8	-7.3
500-599	-39.8	-4.8	9.8	44.7	-34.9	-6.2

600-699	-36.9	-3.6	8.1	47.6	-25.6	-5.0
700-	-39.9	-15.4	16.5	40.3	-26.0	-17.8

[00091] The effects of Omacor® omega-3 fatty acids co-administered with simvastatin was evaluated in a study of 20 patients. The patients were initially treated with 40 mg of simvastatin to establish baseline triglyceride levels between 200 and 499 mg/dL. After baseline triglyceride levels were established, the patients were treated with a combination of 4 grams per day of Omacor® omega-3 fatty acids and 40 mg of simvastatin over an 8 week period.

[00092] As shown in Tables 2 and 3, the administration of a combination of Omacor® omega-3 fatty acids and simvastatin reduced triglyceride, total cholesterol, non-HDL cholesterol and LDL cholesterol in the serum of treated patients relative to baseline (simvastatin treatment alone). In addition, the administration of a combination of Omacor® omega-3 fatty acids and simvastatin increased the levels of HDL cholesterol in the treated patients relative to placebo. Surprisingly, as compared to Omacor® treatment alone, non-HDL cholesterol levels were reduced without an increase in LDL cholesterol levels.

Table 2. Median Baseline and Percent Change from Baseline in Lipid Parameters in Patients with TG Levels 200-499 mg/dL After 4 Weeks

	Baseline (after Administration of Simvastatin 40 mg/day) (mg/dL)	% Change After 4 Wks Administration of Simvastatin 40 mg/day and Omacor® 4g/day.	p-value vs. Baseline
Total Cholesterol	186.7	-13.4	0.0050
Non-HDL-C	146.7	-20.6	<0.0001
HDL-C	40.0	+13.4	0.0879
LDL-C	101.0	-8.7	0.0149
TG	256.7	-36.9	0.0020

Table 3. Median Baseline and Percent Change from Baseline in Lipid Parameters in Patients with TG Levels 200-499 mg/dL After 8 Weeks

	Baseline (after Administration of Simvastatin 40 mg/day) (mg/dL)	% Change After 8 Wks Administration of Simvastatin 40 mg/day and Omacor® 4g/day.	p-value vs. Baseline
Total Cholesterol	186.7	-15	<0.0001
Non-HDL-C	146.7	-22	<0.0001
HDL-C	40.0	+8	0.0846
LDL-C	101.0	-4	0.257
TG	256.7	-23	0.002

[00093] The following formulations may be prepared in accordance with the invention:

Formulation 1:

Ingredient	Mg/capsule
K85EE	1000
Dehydrated ethanol	39.5
Capmul® MCM	20
Simvastatin	20

Formulation 2:

Ingredient	Mg/capsule
K80EE	1000
Dehydrated ethanol	50
Propylene glycol monocaprylate	20
Ezetimibe	5

Formulation 3:

Ingredient	Mg/capsule
K85EE	1000
Glycerol	35
Polyethoxylated castor oil	25
Pioglitazone	15

Formulation 4:

Ingredient	Mg/capsule
EPAX7010EE	1000
Propylene glycol	30
Olive oil	50
Atorvastatin	10

We Claim:

1. A method of blood lipid therapy in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a dyslipidemic agent and natural or synthetic omega-3 fatty acids or pharmaceutically acceptable esters, derivatives, conjugates, precursors or salts thereof, or mixtures thereof, wherein the subject has a baseline triglyceride level of 200 to 499 mg/dl and wherein after administration to the subject the triglyceride level and a non-HDL-C level of the subject are reduced without increasing LDL-C as compared to treatment with the dyslipidemic agent alone.

2. The method of claim 1, wherein the dyslipidemic agent is selected from the group consisting of HMG CoA inhibitors, cholesterol absorption inhibitors, CETP inhibitors, niacin and derivatives, fibrates, bile acid sequestrants, MTP inhibitors, LXR agonists and/or antagonists, and PPAR agonists, antagonists and/or partial agonists/antagonists.

3. The method of claim 1, wherein the dyslipidemic agent is selected from the group consisting of HMG CoA inhibitors.

4. The method of claim 1, wherein the omega-3 fatty acids are present in a concentration of at least 40% by weight as compared to the total fatty acid content of the composition.

5. The method of claim 1, wherein the omega-3 fatty acids are present in a concentration of at least 80% by weight as compared to the total fatty acid content of the composition.

6. The method of claim 1, wherein the omega-3 fatty acids comprise at

least 50% by weight of EPA and DHA as compared to the total fatty acid content of the composition.

7. The method of claim 1, wherein the omega-3 fatty acids comprise at least 80% by weight of EPA and DHA as compared to the total fatty acid content of the composition.

8. The method of claim 1, wherein the omega-3 fatty acids comprise about 5% to about 95% by weight of EPA as compared to the total fatty acid content of the composition.

9. The method of claim 1, wherein the omega-3 fatty acids comprise about 40% to about 55% by weight of EPA as compared to the total fatty acid content of the composition.

10. The method of claim 1, wherein the omega-3 fatty acids comprise about 5% to about 95% by weight of DHA as compared to the total fatty acid content of the composition.

11. The method of claim 1, wherein the omega-3 fatty acids comprise about 30% to about 60% by weight of DHA as compared to the total fatty acid content of the composition.

12. The method of claim 1, wherein omega-3 fatty acids comprise omega-3 polyunsaturated, long-chain fatty acids, esters of omega-3 fatty acids with glycerol, esters of omega-3 fatty acids and a primary, secondary or tertiary alcohol, or mixtures thereof.

13. The method of claim 1, wherein the omega-3 fatty acids comprise EPA and DHA in a ratio of EPA:DHA from 99:1 to 1:99.

14. The method of claim 1, wherein the omega-3 fatty acids comprise EPA

and DHA in a ratio of EPA:DHA from 2:1 to 1:2.

15. The method of claim 1, wherein the omega-3 fatty acids are administered apart from administration of the dyslipidemic agent.

16. The method of claim 1, wherein the omega-3 fatty acids and the dyslipidemic agent are administered together in a unit dose form.

17. The method of claim 1, wherein the dyslipidemic agent comprises simvastatin.

18. The method of claim 1, wherein a LDL-C level of the subject is reduced.

19. The method of claim 1, wherein the triglyceride level of the subject is reduced by about 10% to about 65%, as compared to baseline.

20. The method of claim 1, wherein the non-HDL-C level of the subject is reduced by about 10% to about 55%, as compared to baseline.

21. The method of claim 1, wherein the triglyceride level and the non-HDL-C level in the subject are reduced before 48 weeks of therapy.

22. A method of blood lipid therapy in a subject comprising administering to the subject a pharmaceutical composition comprising an effective amount of a dyslipidemic agent and natural or synthetic omega-3 fatty acids or pharmaceutically acceptable esters, derivatives, conjugates, precursors or salts thereof, or mixtures thereof, wherein a HDL-C level in the subject is increased and a LDL-C level in the subject is reduced as compared to treatment with the dyslipidemic agent alone.

23. The method of claim 22, wherein the dyslipidemic agent is selected

from the group consisting of HMG CoA inhibitors, cholesterol absorption inhibitors, CETP inhibitors, niacin and derivatives, fibrates, bile acid sequestrants, MTP inhibitors, LXR agonists and/or antagonists, and PPAR agonists, antagonists and/or partial agonists/antagonists.

24. The method of claim 22, wherein the dyslipidemic agent is selected from the group consisting of HMG CoA inhibitors.

25. The method of claim 22, wherein the subject has a baseline triglyceride level of 200 to 499 mg/dl.

26. The method of claim 22, wherein the HDL-C level of the subject is increased by about 5% to about 25%.

27. A composition for blood lipid therapy in a subject comprising a fixed dosage form comprising a HMG CoA inhibitor and natural or synthetic omega-3 fatty acids or pharmaceutically acceptable esters, derivatives, conjugates, precursors or salts thereof, or mixtures thereof.

28. The composition of claim 27, wherein the dyslipidemic agent is selected from the group consisting of HMG CoA inhibitors, cholesterol absorption inhibitors, CETP inhibitors, niacin and derivatives, fibrates, bile acid sequestrants, MTP inhibitors, LXR agonists and/or antagonists, and PPAR agonists, antagonists and/or partial agonists/antagonists.

29. The composition of claim 27, wherein the dyslipidemic agent is selected from the group consisting of HMG CoA inhibitors.

30. The composition of claim 27, wherein the composition comprises a solvent system including solubilizers in amounts of less than 25% w/w based on the total weight of the solvent system.

31. The composition of claim 27, wherein omega-3 fatty acids comprise omega-3 polyunsaturated, long-chain fatty acids, esters of omega-3 fatty acids with glycerol, esters of omega-3 fatty acids and a primary, secondary or tertiary alcohol, or mixtures thereof.

32. The composition of claim 29, wherein the HMG CoA inhibitor comprises simvastatin.

33. A method of blood lipid therapy in a subject group comprising administering to the subject group a pharmaceutical composition comprising an effective amount of a dyslipidemic agent and natural or synthetic omega-3 fatty acids or pharmaceutically acceptable esters, derivatives, conjugates, precursors or salts thereof, or mixtures thereof, wherein the subject group has a baseline triglyceride level of 200 to 499 mg/dl and wherein after administration to the subject group the triglyceride level and a non-HDL-C level of the subject group are reduced in a statistically significant amount as compared to a control group treated with the dyslipidemic agent alone without increasing LDL-C in a statistically significant amount as compared to the control group treated with the dyslipidemic agent alone.

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(71) Applicant *(for all designated States except US)*: RELIANT PHARMACEUTICALS, INC. [US/US]; 110 Allen Road, Liberty Corner, NJ 07938 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: RONGEN, Roelof, M., L. [NL/US]; 131 Fairmount Road, East, Caliton, NJ 07930 (US). BOBOTAS, George [US/US]; 1245 North Florida Avenue, Tarpon Springs, FL 34689 (US).

(74) Agents: BERMAN, Richard, J. et al.; ARENT FOX, PLLC, 1050 Connecticut Avenue NW, Washington, DC 20036 (US).

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(54) Title: OMEGA-3 FATTY ACIDS AND DYSLIPIDEMIC AGENT FOR LIPID THERAPY

(57) Abstract: A method and composition for blood lipid therapy by administering to the subject an effective amount of a dyslipidemic agent and omega-3 fatty acids. The method utilizes a single administration or a unit dosage of a combination of dyslipidemic agent and omega-3 fatty acids for the treatment of patients with hypertriglyceridemia, hypercholesterolemia, mixed dyslipidemia, coronary heart disease (CHD), vascular disease, artherosclerotic disease and related conditions, and the prevention or reduction of cardiovascular and vascular events.

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

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U.S. : 514/547

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS, WPIDS, USPATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	Nordoy et al. Effects of simvastatin and omega-3 fatty acids on plasma lipoproteins and lipid peroxidation in patients with combined hyperlipidaemia. Journal of Internal Medicine, 1998, vol. 243, pages 163-170.	1-15, 17-26 and 33 — 16 and 27-32
X	Durrington et al. An omega-3 polyunsaturated fatty acid concentrate administered for one year decreased triglycerides in simvastatin treated patients with coronary heart disease and persisting hypertriglyceridaemia. Heart, 2001, vol. 85, pages 544-548.	1-15, 17-26 and 33
X — Y	Nordoy et al. n-3 fatty acids as supplement to statins in the treatment of patients with combined hyperlipidemia. Essent. Fatty Acids Eicosanoid, Invited Pap. Int. Congr. 4th, 1998, pages 256-261.	1-15, 17-26 and 33 — 1-33
Y	Alaswad et al. Combination drug therapy for dyslipidemia. Current Atherosclerosis Reports, 1999, vol. 1, pages 44-49.	1-33

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (571) 273-3201

Authorized officer
James D. Anderson

Telephone No. 571-272-1600

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INTERNATIONAL SEARCH REPORT

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C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,502,077 (Breivik et al.) 26 March 1996 (26.03.1996), entire document.	1-33